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Breeding wheat for drought adaptation:
Development of selection tools for root architectural traits

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Abstract

A crop's ability to explore the soil profile and extract available water at different depths is largely determined by root system architecture. For instance in wheat (*Triticum aestivum* L.), it has been suggested that a narrow and deep root system can provide better access to deep soil moisture. Such root systems are particularly beneficial for rain-fed regions where crops rely heavily on stored soil moisture at depth, as encountered in the eastern Australian wheat belt. Thus, by targeting desirable root architectural traits, wheat breeders could increase genetic gain for yield in response to the growing demand for food. Yet, selection for these below-ground traits is challenging because roots are difficult to measure and are under complex genetic control. The aim of this project was to develop new phenotypic and molecular selection tools to facilitate selection for root architectural traits in Australian wheat breeding programs targeting terminal moisture stress adaptation. This project focuses on narrow seminal root angle and high number of seminal roots in wheat seedlings; two proxy traits for desirable mature root system architecture. Firstly, to overcome the lack of efficient root screening methods, a high-throughput and cost-effective method for phenotyping seminal root angle and number in wheat was developed, using clear pots in a controlled environment growth facility. Compared to pre-existing phenotyping methods, the newly developed method successfully provided higher heritability, greater repeatability, and better efficiency in terms of time, space, and labour. Further, the clear-pot method revealed a high degree of phenotypic variation for both seminal root traits. Subsequently, to test the ability to introgressed allelic variation for seminal root angle into elite Australian wheat cultivars via phenotypic selection, backcross tail populations for both narrow and wide root angle were developed, using the clear-pot method. Rapid shifts in both population distribution and allele frequency were observed after just two rounds of selection. Further, comparison of the tail populations revealed some genomic regions under selection, for which marker-assisted selection appeared successful. Hence, genetic diversity can be exploited via phenotypic and molecular selection to target desired root system architecture in wheat breeding programs. Finally, to dissect the genetic controls of root traits, a multi-reference nested-association mapping wheat population was developed. In order to identify quantitative trait loci (QTL) relevant to Australian breeders, three genetic backgrounds relevant to the western, southern, and eastern production regions of the Australian wheat belt were used as references. Genome wide association mapping successfully identified a large number of QTL for seminal root angle and number, each with small to moderate effect. This improved understanding of the genetics controlling root traits provides opportunities for marker-

assisted selection to combine desirable root traits for each of the three Australian mega-regions for cereal production. Furthermore, we believe the strategy and outcomes of this project are transferrable to other wheat breeding programs, thus being beneficial not only for Australia, but also for developing countries experiencing similar terminal moisture stress, such as some Indian, South American, and African cropping regions.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-Reviewed Journal Articles

Richard C, Hickey L, Fletcher S, Jennings R, Chenu K, Christopher J. 2015. High-throughput phenotyping of seminal root traits in wheat. Plant Methods **11**, 13.

Conference abstracts

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– incorporated as Chapter 3.

Contributor	Statement of contribution
Cecile Anais Isabelle Richard (Candidate)	Designed clear-pot experiments (100%) Wrote the paper (80%) Edited paper (60%) Statistical analysis (70%)
Dr Lee Hickey	Wrote paper (20%) Edited paper (10%)
Susan Fletcher	Statistical analysis (30%)
Raeleen Jennings	Designed pouch experiments (75%)
Dr Karine Chenu	Edited paper (15%)
Dr Jack Christopher	Designed pouch experiments (25%) Edited paper (15%)

Contributions by others to the thesis

Dr Jack Christopher and Raeleen Jennings conducted the pouch experiments of Chapter 3. Susan Flecther helped with statistical analyses of Chapter 3. Dr Lee Hickey provided strategic advice to develop the backcross populations in Chapter 4. Dr Jack Christopher, Dr Lee Hickey, Dr Karine Chenu, and Dr Mandy Christopher selected reference parents and founders of the multi-reference parent nested association mapping (MR NAM) population. Dr Jack Christopher and Dr Lee Hickey performed initial crosses (F_1) and first generation in the field (F_2) of the MR NAM population. Dr Mandy Christopher and Raeleen Jennings helped sampling the plants and extracting DNA of the backcross populations of Chapter 4 and MR NAM population of Chapter 5. Pr David Jordan and Dr Emma Mace provided fruitful discussion, methodology, and advice for analysing the MR NAM population. Dr Alison Kelly and Dr FA (Fred) van Eeuwijk provided helpful advice and assistance with statistical and genetic analysis. All supervisors critically revised this thesis as a whole prior to submission.

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Keywords

wheat, breeding, drought, root system architecture, high-throughput phenotyping, nested association mapping, quantitative trait loci, genome wide association mapping

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 060412, Quantitative Genetics (incl. Disease and Trait Mapping Genetics), 50%

ANZSRC code: 070305, Crop and Pasture Improvement (Selection and Breeding), 50%

Fields of Research (FoR) Classification

FoR code: 0604, Genetics, 50%

FoR code: 0703, Crop and Pasture Production, 50%

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AGT	Australian Grain Technologies
AgWA	Western Australia Department of Agriculture
BLUEs	Best linear unbiased estimates
BLUPs	Best linear unbiased predictions
CAIGE	CIMMYT-Australia-ICARDA Germplasm Evaluation
CIMMYT	International Maize and Wheat Improvement Center
COP	Coefficient of parentage
CSIRO	Commonwealth Scientific and Industrial Research Organisation
D	Donor
DArT	Diversity array technology
DArTseq	Sequencing-based diversity array technology
Dh	Dharwar Dry
DNA	Deoxyribonucleic acid
DPI Vic	Department of Primary Industries Victoria
Dr	Drysdale
DRO1	*DEEP ROOTING 1*
EGA	Enterprise Grains Australia
G x E	Genotype-by-environment
GRDC	Grains Research and Development Corporation
GS	Genomic selection
GWAS	Genome wide association mapping study
HIFs	Heterogeneous inbred families
ICARDA	International Center for Agricultural Research in the Dry Areas
IWGSC	International Wheat Genome Sequencing Consortium
LD	Linkage disequilibrium
LPB	LongReach Plant Breeders
Ma	Mace
MABC	Marker-assisted backcrossing
MAGIC	Multi-parent advanced generation inter-cross
Ma-NAM	Mace nested association mapping
MARS	Marker-assisted recurrent selection

MAS	Marker-assisted selection
MR-NAM	Multi-reference parent nested association mapping
NAM	Nested association mapping
NILs	Near isogenic lines
ns	Non-significant
PCA	Principal component analysis
Pop1 – Ma/Dr	Mace/Drysdale//Mace
Pop2 – Su/Dh	Suntop/Dharwar Dry//Suntop
Pop3 – Sc/SB	Scout/SB062//Scout
QDAF	Queensland Department of Agriculture and Fisheries
QTL	Quantitative trait loci
RILs	Recombinant inbred line
rNAM	Related nested association mapping
RP	Recurrent parent
RSA	Root system architecture
Sb	SB062
Sc	Scout
Sc-NAM	Scout nested association mapping
SRA	Seminal root angle
SRN	Seminal root number
Su	Suntop
Su-NAM	Suntop nested association mapping
Uni Syd	University of Sydney
UQ	The University of Queensland

Chapter 1
General introduction

"We have heard about some of the great breakthroughs that have been made in medical research, and we were presented with examples of how some new technologies and some of the genomic developments have impacted the lives of hundreds, or, in some cases, thousands of people. We even heard a case where millions of people were affected by some of the new medical technologies recently developed. But if you really want to work in an area of genomics that has the potential to affect the well-being of hundreds of millions of people, work on wheat." Peter Langridge, 2013.

A critical need to boost wheat performance

Wheat (*Triticum aestivum* L.) is arguably the most significant cereal grown worldwide. The three major cereals - maize (*Zea mays* L.), rice (*Oryza sativa* L.) and bread wheat, provide over 50% of the global food supply. Maize is the most cultivated cereal in terms of production, but is often used for animal feed or industrial purposes, such as biofuels and plastics. By contrast, wheat is the single most important source of food for humans. Wheat provides a critical supply of protein for much of the world's population, particularly in developing countries (Langridge, 2013).

The demand for wheat worldwide is expected to grow dramatically in the future. To provide food and feed in a world of nine billion people by 2050, wheat demand is predicted to increase from 720 million tons during 2013-2014 to more than 900 million tons, which corresponds to a total increase of 125% (Alexandratos and Bruinsma, 2012; FAO, 2017). This implies an annual production growth rate of 3.6% from 2014 – 2050, while wheat yields are estimated to be increasing at only 0.9% per year (Ray et al., 2013). Current rates of yield growth and improvement in genetic yield potential are too low to meet predicted future demand (Sayre *et al.*, 1997). With limited arable land available, significant increases in wheat yield performance are required to meet future demand.

Breeding wheat for drought adaptation

Wheat is constantly exposed to environmental stresses that reduce yield and quality. Water availability is a major limiting factor for wheat worldwide. Wheat tends to be grown in environments where water is limiting (Araus *et al.*, 2008). Depending on their intensity and duration, drought events can impact wheat productivity, from yield reduction to a complete failure of the crop. Predicted climate changes, with increased severity and occurrence of drought episodes, further threaten food security worldwide. Hence, improving yield and yield stability where there is limited rainfall is necessary to meet the ambitious targets for future wheat production.

Since the Green Revolution, wheat productivity has greatly improved worldwide, including in water-limited environments. This is due to improved management practices, such as precision farming or crop diversification, and major technological progresses, such as the expansion of irrigation schemes or the development of new breeding technologies. As technological solutions and expansion of the cropping area are probably reaching their limits, plant breeding is one of the sustainable solutions for future increased production. Yet, plant breeding is a slow process, as it generally requires more than a decade to successfully introgress novel genes into adapted germplasm. Hence, new and more efficient breeding technologies are necessary to facilitate rapid and efficient introgression of new traits into elite germplasm.

Breeding methodologies for the future

Physiological breeding

Despite significant increases in wheat productivity using direct yield selection, the rate of genetic progress is slowing down (Fischer and Edmeades, 2010). Yield is a quantitative trait with often modest heritability and subject to unpredictable gene by environment interactions. Breeding for yield *per se* is extremely difficult, particularly in drought prone environments (Jackson *et al.*, 1996). Hence, breeders have started to adopt new strategies based on selection for less complex surrogates to drive faster yield gains.

Physiological approaches are based on proxy traits that have higher heritability and lower G × E interactions than yield. In recent years, many traits associated with drought adaptation have been suggested for incorporation into wheat breeding programs (Richards, 2008;

Reynolds and Tuberosa, 2008). Selection for these proxy traits can be performed in early generations to rapidly eliminate breeding lines with undesired characteristics, prior to the more expensive multi-environment testing of elite lines in the field. These trait-based approaches, together with application of new biotechnologies, can complement yield-based selection to achieve significant genetic gains in yield potential.

Molecular breeding

Technologies used in wheat breeding have shifted towards molecular breeding in recent years. The recent development of low-cost array-based marker systems, such as the sequencing-based diversity array technology (DArTseq), has facilitated high-throughput genotyping for genetic studies. Furthermore, the use of consensus maps in wheat has allowed positioning of markers for construction of genetic linkage maps and comparing results across studies (Akbari *et al.*, 2006). Concurrently, the development of new genetic population designs, such as nested association mapping (NAM) or multi-parent advanced generation inter-cross (MAGIC), has enhanced power, diversity, and resolution for genome wide association studies (Yu *et al.*, 2008; Cavanagh *et al.*, 2008). These new tools have enhanced the ability to detect marker-trait associations for complex polygenic traits, thus offering applications for plant breeding.

The advent of cost-effective whole genome profiling has also contributed to the increasing popularity of marker-assisted selection (MAS) and genomic selection (GS) to reduce delivery times for improved cultivars. High throughput genotyping and genomic breeding are becoming more common in plant breeding. However, these techniques are still reliant on efficient phenotyping methods to identify marker-trait associations. As a result, the collection of phenotypic data is increasingly becoming a limiting factor in breeding programs. Thus, identification of proxy traits and development of high-throughput phenotyping methods are critical to compliment modern genomics and to facilitate molecular breeding.

Root system architecture: opportunities and limitation

Root system architecture (RSA) has been suggested as a drought-adaptive trait in cereal crops including maize, wheat and rice (Ludlow and Muchow, 1990). For instance, crops with deeper roots have better access to water deeper in the subsoil layers (Manschadi *et al.*, 2006; Hund *et al.*, 2009*a*; Lopes and Reynolds, 2010; Henry *et al.*, 2011; Ober *et al.*, 2014). Selection for such root characteristics could enhance crop access to water, enabling plants

to maintain yield under limited rainfall conditions. This would be particularly beneficial in rain-fed systems when rainfall is insufficient to replenish exhausted stores of water in upper soil layers (Manschadi *et al.*, 2006; Hall and Richards, 2013). This would be advantageous to crops grown in environments experiencing terminal drought stress, such as some Australian, Indian, South American and African cropping regions.

While the value of optimum root architectural traits has been demonstrated for drought adaptation, wheat breeders are still reluctant to select for below-ground traits mainly due to the difficulty of phenotyping. Wheat root networks function via complex and dynamic interactions with their below-ground environment. This makes the study of wheat root systems tedious, time-consuming and labour intensive. In recent years, more efficient and accurate phenotyping methods have been developed in the field and laboratory. However, none of these methods are suitable for phenotyping large numbers of individuals as required for effective integration in a large-scale breeding program. Furthermore, the genetic control of root traits is complex, with multiple genes of small effect interacting with each other and with the environment, particularly in water-limited environments (Liu *et al.*, 2013). Hence, despite rapid advances in genomic approaches to tackle complex traits, the lack of large-scale phenotyping methods for root traits remains a major bottleneck to elucidation of the genetic control mechanisms. Thus, development of high-throughput phenotyping methods will be a critical step in introgressing desirable root architectural traits into elite wheat cultivars.

Project objectives

Here, we propose a physiological trait-based approach that could be applied in wheat breeding programs to assist in speeding up the development of drought-adapted cultivars (Figure 1). The strategy targets desirable RSA for wheat yield improvement in terminal moisture stressed environments, using the Australian wheat belt as a case study. The first step is the identification of candidate proxy traits for desirable RSA in target environments. Proxy traits must be highly heritable, and associated with the growth and the functioning of the mature root system. Most importantly, they must be associated with yield or yield components in target environments while offering opportunities for large-scale screening. Once appropriate target traits have been identified, the next step is the development of low-cost and high-throughput phenotyping methods to facilitate selection of these traits in

breeding programs. Breeders can use these phenotyping methods to assess genetic diversity in their germplasm and identify lines with desired RSA for top-crossing and phenotypic selection in segregating generations. The last step is the dissection of genetic control mechanisms for these proxy traits using specific genetic designs such as multi-parental populations. Molecular markers associated with desired proxy traits can then be identified and used to select parental lines or progenies via molecular selection. This approach should lead to the development of phenotypic and molecular selections tools for wheat breeders to facilitate the development of superior genotypes.

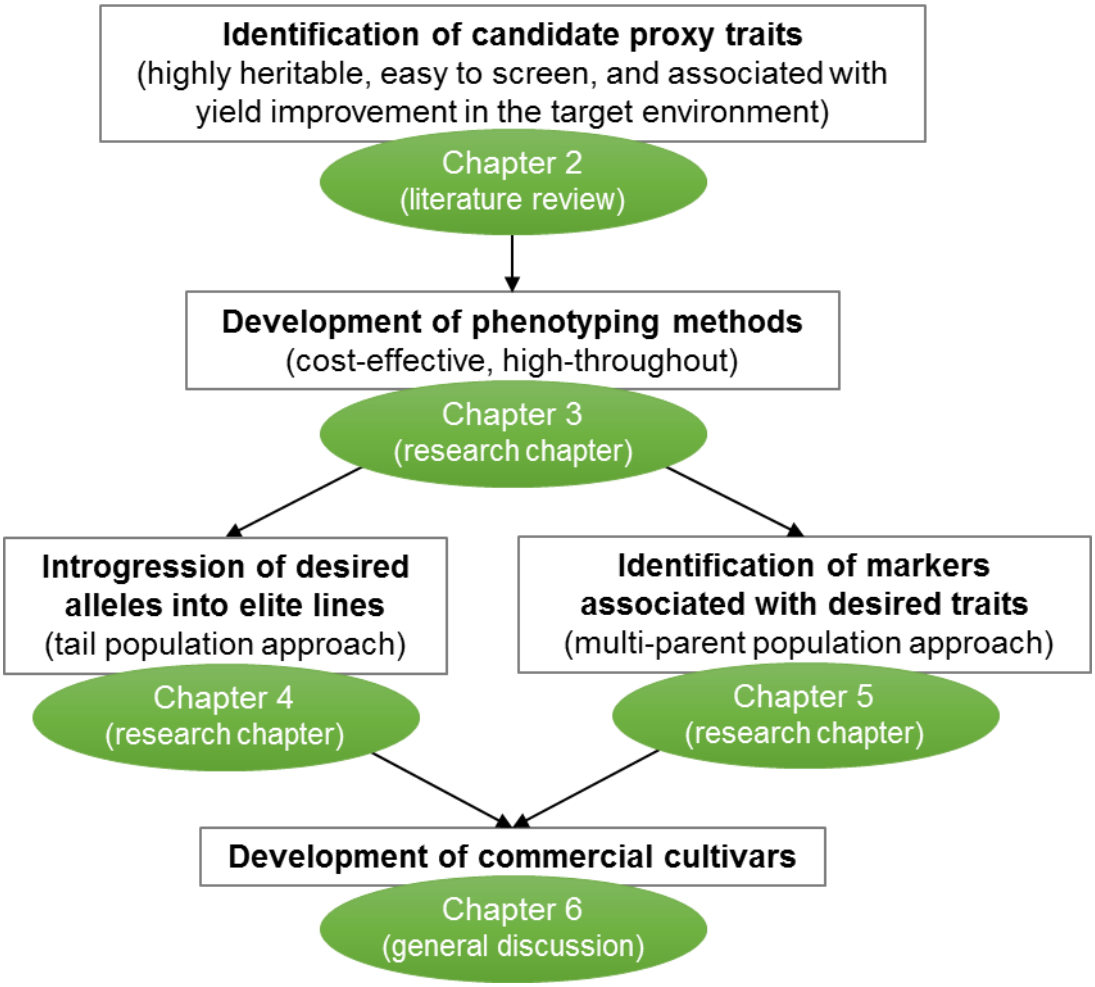

Figure 1: Strategy applied in this study for developing selection tools for wheat breeders to facilitate the development of commercial cultivars

Thesis outline

The thesis is constructed around a literature review (Chapter 2), three core research chapters (Chapter 3 - 5), and a general discussion (Chapter 6), as shown in Figure 1.

The literature review (Chapter 2) concerns the challenges associated with breeding wheat for drought adaptation, and the importance of RSA for drought adaptation. The review highlights why root traits have not been actively selected and how to accelerate the deployment of root architecture genes in breeding programs. Two candidate proxy traits for wheat yield improvement in terminal moisture stress are suggested: narrow seminal root angle (SRA) and high seminal root number (SRN). Both traits are expressed at early growth stages, and have been associated with the spatial root distribution of the mature root system (Nakamoto *et al.*, 1991; Oyanagi *et al.*, 1993, 2001; Nakamoto and Oyanagi, 1994; Bengough *et al.*, 2004; Manschadi *et al.*, 2006; Kato *et al.*, 2006).

The three core research chapters were designed to provide new selection tools allowing wheat breeders to target root traits (Figure 1). Initially, a method for phenotyping SRA and SRN in wheat was designed using clear pots in a controlled environment growth facility (Chapter 3). Using a set of fixed lines, the new method was assessed by comparing results to a pre-existing phenotyping method based on growth pouches, in terms of throughput, repeatability, heritability and opportunity for integration into breeding programs.

The phenotyping method developed in Chapter 3 was then used to test the ability to introgress alleles for narrow SRA into elite wheat lines (Chapter 4). First, an experiment was conducted to characterise genetic diversity in a panel of 22 Australian-adapted wheat lines. Three donor lines for narrow SRA were selected and backcrossed to three Australian wheat cultivars, resulting in three backcross populations. Rounds of bi-directional selection were applied in each population in early generations to develop tail populations for ‘narrow’ and ‘wide’ SRA. Population distributions were compared between tail populations to evaluate the shifts in phenotypic distribution resulting from selection cycles. Finally, tail populations were genotyped using the DArTseq marker platform, and compared via marker frequency analysis to evaluate shifts in allelic frequency, and identify genomic regions influencing SRA in specific populations. Marker-assisted selection for these regions successfully was applied in an independent population derived from the same parental lines to test the effectiveness of molecular selection for SRA.

To allow a more powerful and precise dissection of the genetic control mechanisms of root traits compared to Chapter 4, a multi-reference parent nested association mapping (MR NAM) strategy was applied (Chapter 5). This population was developed by nesting 11 diverse founders within three cultivars for the western, southern, and eastern production

regions of the Australian wheat belt. Recombinant inbred lines were derived using an incomplete factorial design crossing scheme, producing 612 $F_{4:5}$ NAM lines consisting of 15 families. The MR NAM population was genotyped with DArTseq markers and characterised for SRA and SRN using the clear pot phenotyping method developed in Chapter 3. Genome wide association mapping was performed to identify quantitative trait loci (QTL) for root traits and estimate effects related to genetic background.

Information generated in the project will be used to provide recommendations and tools to breeders assisting them to combine desirable root traits for each of the three mega cereal production regions of Australia.

Chapter 2
Literature review

Wheat breeding in Australia

Wheat (*Triticum aestivum* L.) is the most important cereal crop in Australia. Each year, a considerable amount of public funds for research and development are devoted to wheat research. Part of these funds comes from production levies redistributed by organizations such as the Grains Research and Development Corporation (GRDC). There are four main wheat breeding companies in Australia: Australian Grain Technologies (AGT), LongReach Plant Breeders, Intergrain, and HRZ Wheats. All aim to rapidly develop cultivars with improved characteristics to Australian farmers, such as improved adaptation to drought, frost, and salinity, as well as disease resistance to rust, crown rot and nematodes.

Drought adaptation in particular, is a major target for Australian wheat breeders. The Australian wheat belt presents highly diverse rainfall patterns and soil types across the three major cropping regions: the west, south, and east. Sandy-loamy soils with less extractable water predominate in the west, heavy clay-vertosol soils with high soil water retention dominate in the east, while there is much more variability in the south. Rainfall is winter-dominant in the western region, evenly distributed over the year in much of the southern region, and summer-dominant throughout most of the eastern region. This variability leads to a different number and duration of water-stress events in the different regions and in different seasons. Within regions, stresses range from short-term water-deficit, mild water shortage during grain filling relieved by maturity, more severe water stress during the vegetative stage relieved during mid-grain filling, to severe water deficit throughout the grain-setting and the grain-filling periods (Chenu *et al.*, 2013).

Breeders, physiologists, and geneticists, have been working together to unravel the genetics of key traits associated with drought adaptation to accelerate genetic gain for yield under water-limited environments. However, breeding wheat for drought adaptation turns out to be extremely challenging as explained in the following section.

Challenges to breed wheat for drought adaptation

Drought adaptation is a complex trait

Drought adaptation is the ability of a plant to maintain productivity in the face of limited water supply for a period of time. A number of strategies for adaptation to drought have been identified in crops (Levitt, 1980; Tuberosa *et al.*, 2003; Borrell *et al.*, 2006). For instance, crops can avoid dehydration, through greater accessibility to water and lower water loss from the canopy. Alternatively, crops can adapt to dehydration, for example through osmotic adjustment to maintain turgor and hence sustain metabolic activity during drought (Ludlow, 1980). Finally, crops can escape drought, for example through early flowering. Breeders have been focusing on traits associated with these different strategies to improve yield in different water-limited environments. Despite great advances in this area, genetic mechanisms underlying drought adaptation in wheat are still not well understood due to three main factors.

First, complex quantitative traits such as drought adaptation are often influenced by genetic context-dependencies, i.e. the interactions between gene, environment and management (Cooper *et al.*, 2005). A given trait may therefore be beneficial in a specific drought scenario but disadvantageous in others (Tardieu, 2012). For example, early flowering under terminal drought stress allows plants to complete grain filling when water is still available for photosynthetic and remobilization activities. However, early flowering under more favourable conditions can decrease the time available to accumulate biomass leading to lower yield potential (Passioura and Angus, 2010). Given the variability in rainfall patterns over the years, it is of major importance to identify traits that confer improved yield in target environments, while still maintaining high yield potential in seasons without drought (Fleury *et al.*, 2010, Chenu *et al.*, 2011, 2013).

Secondly, drought adaptation studies are often complicated by the effects of confounding factors that affect crop drought stress responses. For example, several types of abiotic stress, such as heat and nutrient deficiencies, can affect plants at the same time as drought, leading to different types of stress adaptive responses. Similarly, variations in flowering time can bring forward or postpone key developmental stages, such as nutrient partitioning and grain filling, causing different types of drought stress responses. Although studying drought adaptation itself has greatly contributed to the development of improved cultivars, interaction between multiple stresses must also be considered to better represent field conditions.

Finally, key traits associated with improved yield under water-limited environments are often under complex genetic control, characterized by low heritability and large genotype-by-environment (G x E) interactions (Jackson *et al.*, 1996). Despite progress in breeding technologies and the identification of numerous quantitative trait loci (QTL) associated with drought adaptation traits, the contribution of such QTL to released cultivars has been modest to date (Richards *et al.*, 2010; Fleury *et al.*, 2010). This is partly due to the QTL approach *per se*: QTL vary across genetic backgrounds, hence QTL information can be difficult to extrapolate from specific mapping populations to other breeding populations. This is also due to the difficulties in implementing marker-assisted QTL selection in breeding programs as explained below. Numerous QTL associated with drought adaptation have been identified in wheat, but they are generally controlled by multiple genes of small effect (Rebetzke *et al.*, 2007, 2014, Bennett *et al.*, 2012*a*, Yang *et al.*, 2007; Dreccer *et al.*, 2013). Hence, given the large number of other traits breeders already need to combine, it is unlikely that markers linked to minor QTL will be targeted, unless they contribute to significant yield increase in most environments and are difficult to phenotype (Richard *et al.,* 2009). Finally, even when major key QTL are identified (Zhang *et al.*, 2014; Li *et al.*, 2014, Bennett *et al.*, 2012*b*), they are often too poorly defined by markers to be useful for breeders (Richard *et al.,* 2009). Hence until now, molecular selection has been limited and breeding has relied more heavily on direct phenotypic selection for traits associated with yield improvement in drought-prone environments.

Wheat has a complex genome

Breeding wheat for drought adaptation has been further limited by the availability and quality of the wheat reference sequence. Wheat has a hexaploid genome (6x = 2n = 42), which formed about 10,000 years ago from hybridization events involving three different species. The predicted closest extant representatives of the ancestral parental diploid species (2n = 14) are *Triticum urartu* (A genome), *Aegilops speltoides* (S genome related to the B genome), and *Aegilops tauschii* (D genome, Dubcovsky and Dvorak, 2007). The three sets of very similar chromosomes resulted in a large genome (AABBDD) of 17 Gb. Moreover, the wheat genome includes highly repeated families and sequences that result from the amplification of transposable elements (Choulet et al., 2010). Due to its large size, polyploidy and repetitive nature, the wheat genome has been challenging to sequence. The International Wheat Genome Sequencing Consortium (IWGSC) has been working since 2005 to develop a physical map and to sequence the individual chromosomes and

chromosome arms of the wheat genome. A chromosome-based draft genome sequence was made available in 2014, followed by the release of the first version of the chromosome-based reference sequence in January 2017. Until the wheat genome was completely sequenced and publicly available, genetic studies made use of consensus molecular marker-based maps to position genes. Some comparative genomics with other cereals with sequenced genomes such as barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), rice (*Oryza sativa* L.), and sorghum (*Sorghum bicolor* L.) or model grass *Brachypodium distachyon*, can also accelerate identification of genes underlying important traits (Gupta *et al.*, 2008).

Keys to improve drought adaptation in wheat

Trait-based approaches

Traditional breeding or selection based on yield *per se*, has significantly contributed to increased wheat yield in water-limited environments. However, grain yield has a low heritability and high G x E interactions under drought conditions. Hence, it is difficult to make genetic progress by selecting for yield *per se*. The use of secondary traits has been suggested to improve the selection response by focusing on direct effects of drought and avoiding confounding factors that contribute to final grain yield (Edmeades et al., 1996; Bänziger, 2000).

Recent studies have shown that trait-based approaches can complement traditional breeding to improve drought adaptation in wheat. For example, selection for above ground traits such as higher transpiration efficiency, greater early vigour and reduced tillering has led to the development of drought-adapted elite lines in Australian wheat breeding programs (Richards, 1996). It is possible that selection for these secondary traits has also contributed to selection for below-ground traits. For example reduced tillering is has been associated with increased root length, root biomass, and root-to-shoot ratio (Hendriks *et al.*, 2016). Trait-based approaches tend to be easier and cheaper than selection for yield itself, and some can be conducted out-of-season. These approaches can help to unravel the physiological and genetic basis of yield formation in cereals, and drive faster yield gains.

Successful application of these trait-based approaches relies on the identification of proxy traits that have higher heritability and are subjected to lower G x E interactions than yield itself. Importantly, these proxy traits must be associated with yield or yield components in many of the growing seasons over a large target area, without being deleterious in seasons

without drought. This insures that the investments in breeding programs are focused on traits offering potential for field advantage in relevant environments.

Drought-adaptive traits

Passioura, (1977) proposed a simple formula that highlights the importance of water uptake and water use for improved water-limited yield. When water is limiting, grain yield is a function of three major components: (i) the water use, i.e. the total amount of water transpired by the crop, (ii) the water use efficiency, i.e. the amount of biomass produced per unit of transpired water, and (iii) the harvest index, i.e. the ratio between grain yield and total biomass. Although this formula does not directly take into account the timing of plant development and water availability, it is likely that improvement to any one component would enhance crop yield in a water-limited environment (Passioura and Angus, 2010).

A wide range of physiological and morphological traits that improve water use and water use efficiency have been identified across crops (Richards, 2008; Reynolds and Tuberosa, 2008). Some shoot-related traits are now being used as secondary selection criteria in breeding programs targeting drought adaptation (Reynolds *et al.*, 2009). For example, cool canopy temperature is associated with greater access to water by roots, and hence greater water uptake (Araus *et al.*, 2002; Trethowan and Reynolds, 2007). By contrast, low carbon isotope discrimination (Rebetzke *et al.*, 2002; Condon *et al.*, 2004), and functional stay-green (Borrell *et al.*, 2014; Christopher *et al.*, 2016) are associated with higher transpiration efficiency, and hence higher water use efficiency. For practical reasons, crop breeding programs have mainly focused on above ground traits (Sinclair *et al.*, 2004; Richards, 2006). However, below-ground traits also offer great opportunities for wheat improvement in drought prone environments (Richards, 2008).

The role of roots in drought adaptation

Root system architecture

The spatial and temporal configuration of the root system in the soil, referred to as root system architecture (RSA), determines the ability of a plant to access water, and is therefore an important aspect for crop productivity and yield stability in water-limited environments (Ludlow and Muchow, 1990; Lynch, 1995). Roots are dynamic, as they respond to changing moisture and nutrient status, temperature, pH, and they interact with organisms present in

the rhizosphere (Bao *et al.*, 2014; Robbins and Dinneny, 2015). Roots are also able to communicate with the above ground part of the plants through complex signalling pathways (Takei *et al.*, 2002; Yoo *et al.*, 2012; Forde, 2014). Thus, root morphology and physiology can impact growth and development of the above ground parts of the plants (DoVale and Fritsche-Neto, 2015). The plasticity of roots in response to environment provides opportunities for exploring natural variation and to identify beneficial root traits to enhance plant productivity (Kano *et al.*, 2011; Grossman and Rice, 2012).

Genotypic variation for root architectural traits and their functional implications for water extraction have been reported for many crop species (O'Toole and Bland, 1987; Ludlow and Muchow, 1990). Recent studies in cereals have suggested a link between some of these root architectural traits and improved yield in drought-prone environments (Kell, 2011; Uga *et al.*, 2013; Narayanan *et al.*, 2014). For instance, higher root length density can increase the rate of water extraction, greater branching can increase the extent of water extraction, while deep rooting is important for water extraction from depth (Price and Tomos, 1997; Courtois *et al.*, 2009; Sadok and Sinclair, 2011; Tuberosa *et al.*, 2011; Varshney *et al.*, 2011; Wasson *et al.*, 2012). Thicker roots can also allow more water to be delivered to shoots, as they tend to have larger diameter xylem vessels (Yambao *et al.*, 1992), which are expected to have higher hydraulic conductivity. A number of specific root architectural traits have been suggested for application in breeding programs to improve yield in drought-prone environments (Wasson *et al.*, 2012; Comas *et al.*, 2013; Brown *et al.*, 2013; Lynch *et al.*, 2014; Lynch and Wojciechowski, 2015). Successful incorporation of these traits depends on the heritability of the trait, ability to accurately and efficiency phenotype the trait, soil properties and target environments (Meister *et al.*, 2014).

Desired root traits for Australia

In sub-tropical, eastern Australia, spring habit wheat sown in autumn, grows over winter and is harvested late in spring. The summer dominant rainfall pattern forces crops to rely heavily on summer rainfall stored in the deep clay soils during the drier winter period. Crops are often exposed to terminal moisture stress, with limited water supply for grain filling (Chenu *et al.*, 2013). Certain wheat cultivars with a deeper root system, a narrower lateral root distribution and a greater root length density at depth, have the ability to extract more soil moisture from deep heavy soils (Asseng and Turner, 2007; Lilley and Kirkegaard, 2011, 2016; Ober *et al.*, 2014). For example, in a study comparing the drought tolerant line SeriM82 with the Australian cultivar Hartog in large soil-filled chambers, SeriM82 was found

to have a narrower root architecture at flowering, and also extracted more soil moisture from deep in the profile (Manschadi *et al.*, 2006). Hence, breeding for deep rooting could enhance access to water, particularly after anthesis.

Improved access to water is particularly beneficial late in the season, when relatively small amounts of subsoil water can have a major impact on grain yield (Kirkegaard *et al.*, 2007). For instance, additional water used after anthesis can be converted to grain at a rate of up to 60 kg ha^{-1} mm^{-1} in wheat field experiments (Kirkegaard *et al.*, 2007) and in crop simulations (Manschadi *et al.*, 2006; 2010; Veyradier *et al.*, 2013). Additional water used after anthesis also increased sorghum production by up to 50 kg ha^{-1} mm^{-1} in field trials (Borrell *et al.*, 2014). Field experimentation combined with crop modelling simulations suggest that deep rooting in wheat leads to higher yield in most seasons in eastern Australia, with evidence of stored moisture at depth (Manschadi *et al.*, 2010; Veyradier *et al.*, 2013; Lilley and Kirkegaard, 2016). Importantly, deep rooting may also improve yield in temperate and Mediterranean regions of Australia in some seasons and is rarely associated with lower yield in good seasons (Manschadi *et al.*, 2010; Veyradier *et al.*, 2013; Lilley and Kirkegaard, 2016). Hence, selection for root system with more roots at depth seems highly desirable in each of the three major cropping regions of Australia.

Limitations to breeding for root architectural traits

Phenotypic selection

A current challenge for breeders targeting the roots is the limited ability to phenotype them due to their underground location and large phenotypic plasticity. Until now, RSA of major crops has been mostly indirectly modified by domestication and breeding, toward distribution of roots in the soil that improve water and nutrient uptake in the target agricultural systems. For example, in a crop modelling study, the continuous yield increase of maize in the U.S. corn-belt since 1930 was partly explained by changes in root system architecture, with modern cultivars capturing an additional 270 mm of water throughout the season (Hammer *et al.*, 2009). Similarly in barley, modern cultivars were able to explore larger areas in the soil by producing higher numbers of seminal roots than their earlier counterparts (de Dorlodot *et al.*, 2007). Given that RSA has been indirectly modified during breeding for improved yield, it is likely that a more direct phenotypic selection would allow breeders to develop breeding lines with desired root characteristics more rapidly. Some successful

examples are found in bean (*Phaseolus vulgaris*), where RSA has been directly selected to develop cultivars with shallow root systems to access phosphorus in the surface soil (Liao *et al.,* 2001; Lynch, 2011; Lynch and Brown, 2001). Similarly in rice, a deep rooting line was successfully developed to enhance nitrogen and water uptake (Arai-Sanoh *et al.*, 2014; Uga et al., 2013).

In recent years, more efficient and accurate phenotyping methods have been developed in the field, glasshouse and lab (Table 1; Zhu *et al.*, 2011; Paez-Garcia *et al.*, 2015). Historically, root traits were measured in the field by destructive soil sampling, such as soil coring or by growing plants in mesh bags that were later extracted from the soil (Neill, 1992). New methods have been developed to increase the throughput, such as hydraulic coring and 'shovelomics' (Trachsel *et al.*, 2011; Wasson *et al.*, 2014). However, these approaches are destructive so that integration with selection for other traits or retaining seed of the selected plants is difficult. Moreover, these methods are tedious, time-consuming, labour intensive and are not suitable for fast and cheap screening of large breeding populations.

Table 1: Examples of recent methods for root phenotyping in the field or under controlled environment conditions

Method	Growth media	Description
Growscreen-Rhizo	Soil (lab and glasshouse)	Plants grown in soil-filled chambers (rhizotrons) are characterised in two dimensions for root geometry and temporal growth responses using an automated platform (Nagel *et al.*, 2012; Ytting *et al.*, 2014).
Hydraulic coring	Soil (field)	A tractor equipped with hydraulic soil corer inserts tubes into the soil to sample at different depth (Wasson *et al.*, 2014).
Minirhizotrons	Soil (field)	Transparent tubes inserted into the soil provide direct and non-destructive access to the root system in the field. Roots growing around the outside walls of the tubes can be imaged with cameras inserted down the tube length (Ao *et al.*, 2010; Maeght *et al.*, 2013).
Rhizoponics	Liquid media (lab and glasshouse)	Plants are growing until maturity in a tank filled with liquid media, allowing non-destructive two dimensional imaging of root architecture (Mathieu *et al.*, 2015).
Rhizoslides	Paper-based (lab and glasshouse)	Seeds are growing on moistened germination paper, covered by a plexiglass sheet. The system allows

		separation of crown roots from embryonic roots (Le Marie *et al.*, 2014).
Shovelomics	Soil (field)	Direct and accurate observation of the root system of excavated and cleaned adult plant root systems placed on a phenotyping board (Trachsel *et al.*, 2011). New algorithms allow high throughput extraction of root traits (Bucksch *et al.*, 2014)
X-ray computed tomography	Soil (lab and glasshouse)	A series of X-ray generated projections are acquired to reconstruct the three dimensional spatial distribution of root systems of plants grown in pots (Mairhofer *et al.*, 2013). This non-destructive method has been used to study root-soil interactions *in situ* (Mooney *et al.*, 2012).

Alternatively, root systems can be indirectly evaluated in the field through surrogate traits measured on the above ground part of the plant. For example, the canopy temperature depression of wheat has been partly associated with soil moisture extraction and root depth. Canopy temperature depression can be assessed in the field using a portable infrared thermometer (Lopes and Reynolds, 2010) or a high-throughput thermography aerial systems (Furbank and Tester, 2011; Chapman *et al.*, 2014). These methods are non-destructive, but tend to be expensive and highly influenced by the environment (Leigh *et al.*, 2006).

To facilitate access to the root system in a more homogenous environment, laboratory-based methods have been developed with different growth media, such as liquid culture (Miyamoto *et al.*, 2001), gel (Iyer-Pascuzzi *et al.*, 2010) or growth paper (Hund *et al.*, 2009*b*; Le Marie *et al.*, 2014). These techniques enable root systems to be rapidly and accurately characterised for various traits, but do not take into account the ability of roots to respond to the soil environment, particularly the heterogeneous distribution of nutrient and water in the soil (Hodge, 2004). For example, G x E interactions significantly affect the root length of wheat cultivars grown in sandy soil compared to agar plates (Gregory *et al.*, 2009).

Methods using soil as a growth media have also been developed to come closer to field conditions, such as soil-filled chambers (Manschadi *et al.*, 2008) or rhizotrons (Nagel *et al.*, 2012; Ytting *et al.*, 2014). However, these methods are limited by the difficulty to account for the natural interactions found in the rhizosphere (Passioura, 2006). For example, plant growth promoting rhizobacteria (PGPR) and mycorrhizal fungi can enhance plant growth by

regulating nutritional and hormonal balance, producing plant growth regulators, solubilizing nutrients and inducing resistance against plant pathogens (Nadeem *et al.*, 2014). Additionally, the containers are usually too small to mimic the available soil volumes in the field (Poorter *et al.*, 2012). Therefore, characterisation of root traits under controlled environment conditions at early developmental stages may not be directly associated with phenotypic expression in the field at later growth stages (Passioura, 2010).

Phenotyping in the field and controlled environment conditions are complimentary approaches. Root studies performed in the glasshouse or in the lab can be limited in their ability to reproduce field-like conditions (Passioura, 2006, 2010; Poorter *et al.*, 2012). Yet, phenotyping in controlled environment conditions is generally less laborious and less time-consuming than in the field, and can be conducted out-of-season. In addition, root measurements tend to be more precise and more reproducible because the plants are grown in a more homogeneous environment compared to the field. Despite many innovations in root phenotyping over the last few years (Paez-Garcia *et al.*, 2015; Kuijken *et al.*, 2015), suitable methods for large breeding populations are still needed.

Molecular selection

Over the past few years, the advent of low-cost high-throughput molecular markers has facilitated dissection of genetic control for traits relevant for crop productivity. QTL mapping has been successfully conducted to identify the genetic regions associated with drought adaptation (Fleury *et al.*, 2010). However, roots have attracted less attention in genetic studies. This is due to difficulties with measurement *in situ* as discussed above and large phenotypic plasticity (Passioura, 1983; Yu *et al.*, 2006; Bengough *et al.*, 2006; Ito *et al.*, 2006; Lynch, 2007). The lack of high-throughput and large-scale phenotyping methods still remains a major bottleneck to elucidation of the genetic control for root traits.

A few studies have been conducted to identify the genetic regions controlling specific root traits in barley (Robinson *et al.*, 2016), maize (Zurek *et al.*, 2015; Pestsova *et al.*, 2016), rice (Uga *et al.*, 2011; Liang *et al.*, 2013), sorghum (Mace *et al.*, 2012; Rajkumar *et al.*, 2013), and wheat (Sharma *et al.*, 2011; Hamada *et al.*, 2012; Bai *et al.*, 2013; Christopher *et al.*, 2013; Zhang *et al.*, 2014). In some studies, root traits have been associated with yield and yield components under water-limited environments. For instance in rice, a QTL for deep rooting *DEEP ROOTING 1* (*DRO1*) was identified (Uga *et al.*, 2011) and recently cloned in a shallow-rooting rice cultivar to enhance its yield under drought conditions by increasing

deep rooting (Uga *et al.*, 2013). In sorghum, genetic association between narrower nodal root angle and increased grain yield has been found, and manipulating nodal root angle through molecular breeding has been proposed to improve drought adaptation (Mace *et al.*, 2012). In maize, QTL for the root traits primary root length, primary root diameter, primary root weight, and weight of the adventitious seminal roots and some of these QTL are overlapping with QTL for grain yield in the field (Tuberosa *et al.*, 2002*b*). The successful identification of QTL for root traits presents new opportunities for selecting desired root architecture and improving drought tolerance in crop breeding programs via genomics-based approaches.

Breeders can apply molecular selection to track and select the genes of interest during crossing and selection in breeding programs. Marker-assisted selection (MAS) has been successfully applied in wheat breeding programs for simple traits (Dubcovsky, 2004; Kuchel *et al.*, 2007; Collard and Mackill, 2008). For example, bacterial blight resistance was successfully introgressed into rice (Joseph *et al.*; Chen *et al.*, 2001), and yellow mosaic virus resistance into barley (Werner *et al.*; Okada *et al.*, 2004). However, when the number of QTL to be manipulated is high, MAS is no longer feasible. As a consequence, MAS still remains limited when many alleles of small effect are involved in the trait of interest (Moreau *et al.*, 2004). Major QTL have been identified for root traits (Price and Tomos, 1997; Giuliani *et al.*, 2005; Uga *et al.*, 2011). However, most of the genetic variation for root traits is driven by minor genes with small effects, interacting with each other and the environment (Tuberosa *et al.*, 2002*a*; Malamy, 2005; Christopher *et al.,* 2013). For example, Liu *et al.* (2013) have identified a total of 52 QTL for six different root traits in wheat under two water regimes, including maximum root length, seminal root number, total root length, project root area, root surface area, and seminal root angle. The study revealed that the QTL were environment-specific and subject to pleiotropic effects. Therefore, new strategies and breeding technologies are required to unravel the genetic basis of these complex traits and facilitate their selection in breeding programs.

Accelerating the deployment of root architectural genes

New strategies and breeding technologies are now available to facilitate deployment of desirable root characteristics in wheat. This includes the identification of proxy traits for desired root characteristics, new genetic designs, statistics, and modelling to dissect

complex traits, as well as genomics-based methods to facilitate selection of these complex traits in breeding programs.

Identification of proxy traits

Root system architecture of a mature plant may be associated with proxy traits measured in seedlings, providing an opportunity for large-scale and cost-effective screening. For instance in oilseed rape (*Brassica napus* L.), seedling root architectural traits measured in controlled environment conditions were linked to variation in seed yield and nutrient capture in the field (Thomas et al., 2016). Two types of roots occur in cereals, the seminal roots coming directly from the embryo and the later, nodal roots, emerging at the lower tiller nodes (Manske and Vlek, 2002). A number of studies for several species have reported that the angle between the first pair of seminal roots, the seminal root angle (SRA), as well as seminal root number (SRN), were associated with the 3D growth and functioning of the root system later in the season, thus affecting the timing and amount of water uptake (Nakamoto *et al.*, 1991; Oyanagi *et al.*, 1993, 2001; Nakamoto and Oyanagi, 1994; Bengough *et al.*, 2004; Manschadi *et al.*, 2006; Kato *et al.*, 2006). For instance in wheat, characterisation of SRA for 27 cultivars revealed that those adapted to drought-prone environments relying on soil moisture stored at depth were more likely to have a narrow growth angle and a deeper root system, as opposed to the cultivars adapted to Mediterranean environments (Manschadi *et al.*, 2008). In other cereals, the association between seminal root traits and deeper, more branching rooted systems has been demonstrated in sorghum, maize and rice (Singh *et al.*, 2010; Uga *et al.*, 2011). Additionally, a number of QTL showing homology across species have been reported recently (Mace *et al.*, 2012). SRA and SRN are expressed at an early developmental stage, offering opportunities for large-scale screening. Hence, narrow SRA and high SRN have been proposed as secondary selection criteria in wheat breeding programs to target improved water use at depth and adaptation to target cropping environments (Manschadi *et al.*, 2010; Wasson *et al.*, 2012; Casadebaig *et al.*, 2016).

New genetic designs

In recent decades, traditional genetic studies, such as linkage analysis and association mapping strategies, have been commonly used to dissect the genetic basis of these complex traits (Mackay, 2001; Hackett, 2002; Gupta *et al.*, 2005). Association mapping uses historic recombination events in germplasm collections or natural populations to identify QTL associated with a trait, while linkage analysis uses recent recombination events in large

families derived from two parental lines. The two methods are complimentary. Compared to linkage analysis, association mapping targets broader genetic variations by assessing a large number of polymorphic loci and multiple alleles at each locus whereas in linkage analysis fewer polymorphic loci and only two alleles at each locus can be detected. Thus, association mapping generally offers higher resolution mapping due to a greater number of recombination events. However, association mapping has a lower power to detect the effect of rare alleles compared to linkage analysis which has a higher statistical power due to greater allele replication. In addition, association mapping requires more markers to get genome wide coverage and false associations are commonly detected due to population structure. Recently, new genetic designs have been developed to help overcome these limitations.

The nested association mapping (NAM) strategy has been developed to combine the power of linkage analysis with a defined population structure and the high resolution of association mapping with greater genetic diversity (Stich, 2009). A NAM population is obtained by crossing a panel of founder lines to a reference variety, and producing a number of recombinant inbred lines (RILs) from each cross. The RILs are characterised as 'mosaics' of chromosomal segments from the different donors in the same background (Stich, 2009). The NAM strategy was originally developed in maize (Yu and Buckler, 2006) and was applied to demonstrate that large differences in flowering time in maize were caused by cumulative effects of numerous QTL rather than few genes with large effect (Buckler *et al.*, 2009). NAM has been rapidly recognised as a broadly relevant approach and platforms are in development for other major crop species including sorghum (Jordan *et al.*, 2011; Mace *et al.*, 2013), barley (Schnaithmann *et al.*, 2014; Maurer *et al.*, 2015; Saade *et al.*, 2016), soybean (*Glycine max* L.*,* Guo *et al.*, 2013), and wheat (Bajgain *et al.*, 2016)

The multi-parent advanced generation inter-cross (MAGIC) strategy has also been proposed to combine advantages of linkage analysis and association mapping with low marker density requirements, high allele richness, high mapping resolution, and high statistical power (Cavanagh *et al.*, 2008). MAGIC populations are created by inter-crossing multiple lines until all founders are combined with equal proportions in the inter-crosses and then deriving the subsequent RILs. The MAGIC strategy has been used in wheat for mapping QTL underlying complex traits such as plant height and grain hectolitre weight (Huang *et al.*, 2012). MAGIC populations are in development for other crop species,

including rice (Bandillo *et al.*, 2013; Meng *et al.*, 2016) and wheat (Cavanagh *et al.*, 2008; Mackay *et al.*, 2014; Delhaize *et al.*, 2015).

A comparison among different types of bi-parental and multi-parental populations, including MAGIC and NAM populations, was presented by Bohra (2013). In the last decade, the use of these new genetic designs along with the development of high-throughput low-cost molecular marker systems has facilitated detection of QTL for complex polygenic traits (Ehrenreich *et al.*, 2009; Brachi *et al.*, 2010; Kump *et al.*, 2011; Tian *et al.*, 2011). These new multi-parent populations offer great opportunities to dissect complex traits such as root system architectural traits.

Statistics and modelling

Dissection of complex traits can also be supported by the development of statistical and modelling methods. Statistical approaches can improve the power to accurately detect QTL and estimate their effects. For example, a mixed linear model approach that takes multiple QTL and QTL by environment interactions into account for mapping QTL has been developed (Qi *et al.*, 2014). Crop simulation models can also be used to extrapolate the phenotypic data obtained from field and controlled environment studies to a wider range of environments (Chenu *et al.*, 2009). This approach can aid in quantifying the value of traits in the target environments (Manschadi *et al.*, 2006) and help to analyse trait values in multi-environment trials (Chenu *et al.*, 2011; Christopher *et al.*, 2013). For example, modelling studies for drought adaptation in wheat and sorghum have evaluated the effect of specific traits and combinations of traits on yield, in interaction with the crop growth and the environment (Chapman, 2008). In addition, the value of traits in breeding, over several cycles of selection, can be evaluated when combining breeding-system and crop models (Cooper *et al.*, 2005; Chapman, 2008). Such modelling studies provide quantitative information on how the traits would likely be selected over time, in particular when different environments are sampled (Chapman *et al.*, 2003).

Genomics-based methods

In recent years, the use of molecular markers has facilitated selection for traits underpinned by major genes such as disease resistance and some quality traits (Dubcovsky, 2004; Kuchel *et al.*, 2007). Once markers associated with QTL of interest are identified, the next step is their deployment in breeding practices. MAS has been successfully applied in wheat for simple traits underpinned by major genes (Ellis *et al.*, 2002; Mago *et al.*, 2005; Lagudah

et al., 2006). However, for more complex polygenic quantitative traits, marker-assisted backcrossing (MABC), marker-assisted recurrent selection (MARS), and genomic selection (GS) are more appropriate (Bernardo, 2008). MABC leads to the development of superior genotypes that contain major QTL from one source (a donor parent), while retaining the whole genome of the recurrent parent. MARS involves successive inter-crossing of selected individuals, and allows incorporation of desirable alleles from different sources into elite lines. Unlike MABC or MARS that usually relies on major QTL, GS takes into account all molecular markers affecting the trait, including those with small effects, and would therefore be more suitable for complex traits such as drought adaptation. In this approach, genomic estimated breeding values are calculated from individual lines in training populations based on phenotyping and genome-wide marker coverage (Heffner *et al.*, 2009). The breeding values can then be applied to select progeny based on marker data only, prior to phenotypic evaluation.

Conclusion

Root architectural traits have great potential to enhance productivity under water deficit. In the last decade cheaper and faster sequencing methods have resulted in an enormous increase in genomic data. Molecular tools are no longer the bottleneck for genetic studies. However, genetic studies on root traits are being impeded by the complexity of these traits and the inaccessibility of the rhizosphere. As a result, the genetic mechanisms underlying root traits is still not well understood, and breeders are limited in their ability to select for desired root system architecture. The development of high-throughput and cost-effective phenotyping methods to characterize below-ground traits will be critical in improving genetic resolution. A better understanding of root responses to their dynamic and heterogeneous environments is also required to adapt breeding programs to specific target environment, as well as identifying root traits that confer a yield advantage during drought episodes while maintaining yields during the good seasons. Great advances have been achieved to understand root traits in maize, rice and sorghum and are promising for breeding wheat. Thus, this study was conducted with aim of developing methods for selecting desired root traits in wheat breeding programs.

Chapter 3
High-throughput phenotyping of seminal root traits in wheat

Abstract

Root system architecture can influence water use and water uptake, yet selection for root architectural traits in breeding programs has been limited by a lack of suitable phenotyping methods. The aim of this chapter was to develop a novel low-cost high-throughput phenotyping method to facilitate selection for desirable root architectural traits. In this chapter, an innovative method based on clear pots was developed to assess the angle and number of seminal roots in seedlings of wheat (*Triticum aestivum* L.) – two proxy traits associated with the root architecture of mature wheat plants. A panel of 24 wheat cultivars was assessed for seminal root angle and number. Results were compared to a pre-existing phenotyping method based on growth pouches. Both methods revealed genetic variation for seminal root angle and number in the panel. The clear pot method developed in this chapter provided higher heritability and higher phenotypic correlations across experiments compared to the growth pouch method. In addition, the clear pot method was more efficient – requiring less time, space, and labour compared to the growth pouch method. Therefore the clear pot method was considered the most suitable for large-scale and high-throughput screening of seedling root characteristics in crop improvement programs. The clear-pot method could be easily integrated in breeding programs targeting drought adaptation to rapidly enrich breeding populations with desirable alleles. For instance, selection for narrow root angle and high number of seminal roots could lead to root systems with higher proportion of roots and more branching at depth. Such root characteristics are highly desirable in wheat to cope with anticipated future climate conditions, particularly where crops rely heavily on stored soil moisture at depth, including some Australian, Indian, South American, and African cropping regions.

Introduction

Aspects of root system architecture can influence the water status of the plant by increasing the rate of water uptake or increasing the amount of water extracted. For instance in wheat (*Triticum aestivum* L.), crops with deeper roots and greater distribution of roots at depth

have better access to water in the deep subsoil layers (Manschadi et al 2006; Lopes and Reynolds, 2010; Ober *et al.*, 2014). Such root characteristics facilitate improved access to soil moisture, particularly late in the season when marginal water-use efficiency for grain production is higher (Manschadi *et al.*, 2010; Lilley and Kirkegaard, 2011). Selection for these root architectural traits would be highly advantageous to crops in rain-fed systems, when there is not enough rainfall to replenish upper soil layers but soil moisture remaining at depth (Manschadi et al 2006; Hall and Richards, 2013).

Despite playing a key role in drought adaptation, root architectural traits have been largely neglected in crop breeding programs due to the difficulty of making measurements *in situ*, lack of efficient root screening methods, low heritability, and large phenotypic plasticity (Passioura, 1983; Yu *et al.*, 2006; Bengough *et al.*, 2006; Ito *et al.*, 2006; Lynch, 2007). In the recent years, methods for phenotyping root traits have been developed in controlled environment conditions and in the field (Paez-Garcia *et al.*, 2015; Kuijken *et al.*, 2015). However, most of these approaches are low-throughput, expensive, and thus poorly adapted for application in breeding programs. Hence, development of high-throughput low-cost phenotyping methods is a critical step to incorporate selection for below-ground traits into breeding programs.

Narrow seminal root angle (SRA) and higher seminal root number (SRN) are proxy traits for a more compact root system with more roots at depth (Manschadi *et al.*, 2006; 2008, 2010; Wasson *et al.*, 2012; Christopher *et al.*, 2013). Methods for measuring seminal root traits in wheat have been developed in the laboratory, using gel-filled chambers (Bengough *et al.*, 2004; Manschadi *et al.*, 2006) or growth pouches (Barker *et al.*, 2006). However, these methods are low-throughput and unadapted for characterising large number of individuals as required in breeding programs.

In this study, we used a panel of 24 spring wheat cultivars to design and evaluated a high-throughput method based on clear pots for measuring SRA and SRN in controlled environment growth facilities. We compared the new method to a pre-existing method based on growth pouches in terms of heritability, repeatability, and efficiency. We discuss the advantages and disadvantages of these root trait phenotyping methods, along with the opportunity to exploit high-throughput phenotypic screening in breeding populations.

Materials and methods

A panel of wheat cultivars differing in their geographic region of adaptation and drought adaptation were assayed for SRA and SRN in clear pots and growth pouches. In total, four experiments were conducted in this study – two based on clear pots (i.e. Clear_1 and Clear_2) and two based on growth pouches (i.e. Pouch_1 and Pouch_2) to assess the robustness and repeatability of each method.

Clear pot method

Two experiments using the clear pot method (Clear_1 and Clear_2) were conducted successively under the same conditions to evaluate the panel of 24 wheat cultivars for SRA and SRN.

Wheat seedlings were cultured in 4 L clear pots (ANOVApot®, 200 mm diameter, 190 mm height, http://www.anovapot.com/). The clear pots were filled with a pine bark potting medium (70% composted pine bark 0–5mm, 30% coco peat, pH 6.35, EC = 650 ppm, nitrate = 0, ammonia < 6 ppm and phosphorus = 50 ppm). Seeds were sown at a depth of 2 cm every 2.5 cm along the pot wall, providing a density of 24 seeds per pot (600 plants / m^2). The seeds were carefully placed vertically, embryo downwards and facing the wall to facilitate root growth along the transparent wall (Figure 2, A). After sowing, the clear pots were placed inside 4 L black pots (ANOVApot®, 200 mm diameter, 190 mm height) to exclude light from the developing roots (Figure 2, B). The pots were watered after sowing and no additional water or nutrients were supplied thereafter.

Figure 2: Wheat seedlings phenotyped for seminal root traits in a high-throughput system using clear pots
(A) Wheat seedlings grown in clear pots under controlled environment conditions (picture taken five days after sowing). (B) The clear pots placed inside black pots to exclude light (picture taken at 11 days after sowing). (C) Images recorded for each plant of each pot using a camera fixed on a tripod, a black box with anti-reflection walls and a revolving stand.

The two experiments used randomised complete block designs where 10 plants of each of the 24 cultivars were randomised to each of 10 pots. Each pot containing one seedling of each cultivar represented one replicate block with one plant of each cultivar representing the experimental unit. The two experiments were conducted in a walk-in, temperature-controlled growth facility. Constant temperature (17°C ± 2 C) was adopted over 24 hours with diurnal (12 hour) natural light.

Five days after sowing, images of the seminal roots visible through the clear wall were recorded using a camera (Canon PowerShot SX600 HS 16MP Ultra-Zoom Digital Camera) fixed on a tripod (Slik F153 Tripod) (Figure 2, C). Images were recorded for each plant by rotating the pot 15^{o} in a clockwise direction. The images captured from each pot displayed some overlap and were joined together to create a panoramic image for the whole pot with the stitching software *PhotoStitch* (http://en.softonic.com/s/photo-stitch, Figure 3, A). This step reduced the picture file storage size and also improved image analysis speed by using one picture per pot instead of 24. Colours of panoramic images were inverted to enhance the contrast between roots and soil, with the software *imageJ* (http://rsb.info.nih.gov/ij/; Schneider *et al.*, 2012), facilitating root-trait measurements (Figure 3, A). For each plant, the growth angle between the first pair of seminal roots was measured at approximately 3 cm distance from the seed (Figure 3, B).

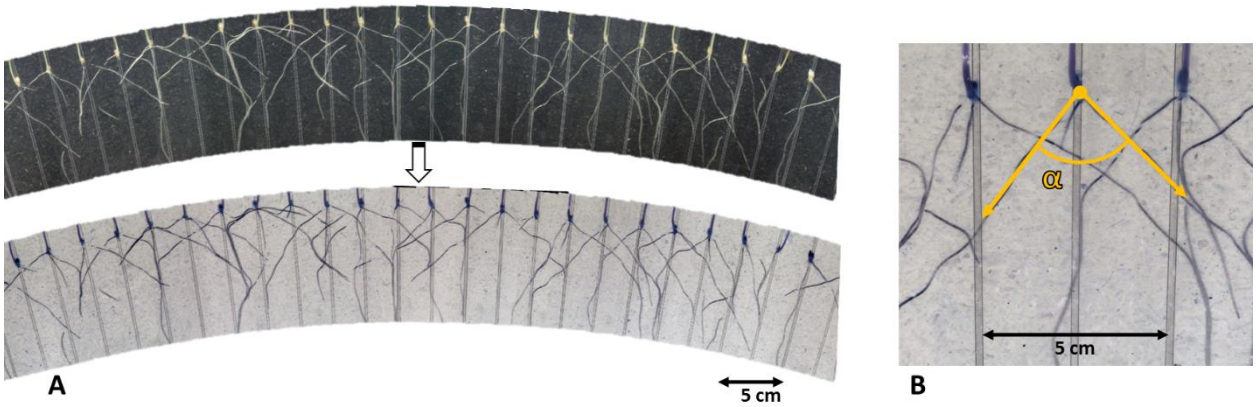

Figure 3: Measuring seminal root angle with the clear pot method

(A) Panoramic image of wheat seedling grown in the clear pot system obtained by stitching images of individual plants using software (*PhotoStitch*) and colours inverted to facilitate root identification. (B) For each plant, the angle (α) between the first pair of seminal roots was measured at approximately 3 cm distance from the seed using software (ImageJ).

In this study, we tested two different ways to measure SRN. The ‘imaged’ SRN was measured based on the photographic images by counting the number of roots emerging from the seed visible through the clear pot. The ‘extracted’ SRN was measured after pulling out the wheat seedlings and counting the number of roots. The number of roots was measured at 11 days after sowing, when most of the genotypes had produced a second pair of seminal roots, but before the network of roots became too complex to facilitate counting through images or by pulling out the plants without damaging the root system.

Growth pouch method

Two experiments (Pouch_1 and Pouch_2) were conducted successively under the same conditions to evaluate the panel of 24 wheat cultivars for SRA and SRN using the growth pouch method.

The experiments were performed using Cyg germination growth pouches (Mega International). Measuring 18 cm × 16.5 cm, the plastic pouches contain perforated germination paper that has been folded to form a continuous trough along the top of the pouch, in which seeds are supported (Figure 4, A). To avoid roots spatially interfering with each other during the initial growth period, each pouch contained only two seeds (Figure 4, A). Pouches were pre-prepared by removing excess paper from the seed trough, leaving two individual troughs (Figure 4, A). Tap water (15 mL) was added to each pouch and allowed to evenly distribute over the germination paper. Dry seeds were placed vertically into the troughs, with the embryo end pointing down, and the embryo facing out towards the plastic. Pouches were then placed vertically into containers, sandwiched between foam to maintain even pressure on the seeds and to reduce air spaces. Containers were covered in cling wrap to prevent moisture loss. Pouches were placed into a plant growth cabinet at a constant temperature of 15^{o} C with no light. After 12 days, lights were turned on using a 12 h photoperiod. Seedlings were grown for 20 days in total.

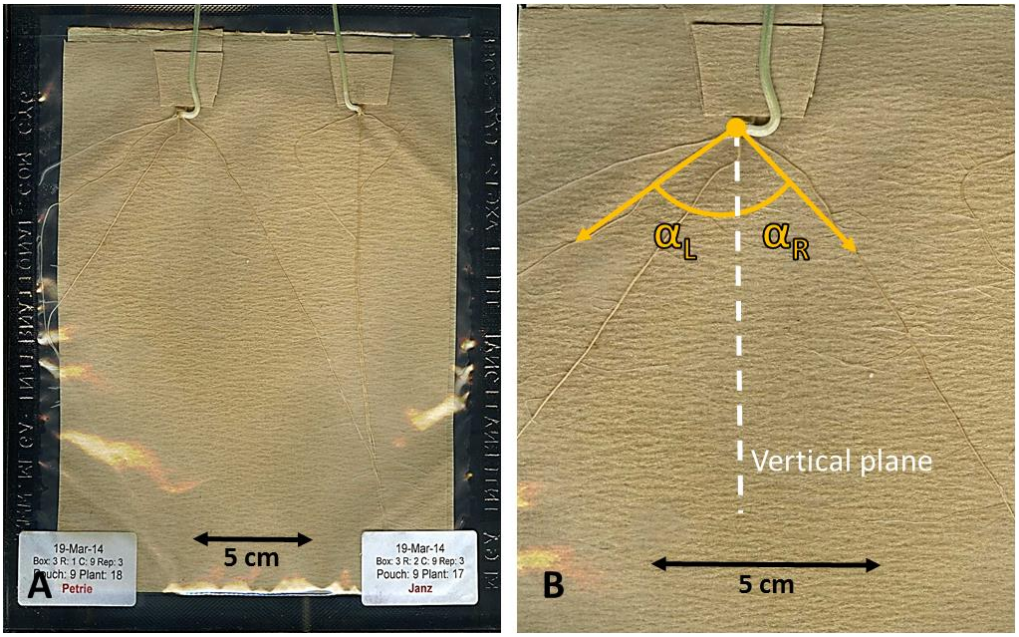

Figure 4: Illustration of a growth pouch

(A) Wheat seedlings were phenotyped for seminal root traits using growth pouches (picture taken 20 days after sowing). (B) For each plant, the left (αL) and the right (αR) angle between each of the first pair of seminal roots and the vertical plane was measured at approximately 3 cm distance from the seed using software (*Opengelphoto*).

The pouch experiments used a resolvable block design where pouches constituted a block size of two. This ensured pairs of cultivars were not in the same pouch together more than once. Each experiment had six boxes with 16 pouches in each box set out in a 2 × 8 array. Each box comprised a replicate block, with one replicate of the panel of 24 cultivars, one extra replicate for Hartog and SeriM82, and one replicate of six other cultivars. The randomisations for the pouch experiments were latinised.

SRA and SRN were measured using a scanner (Epson Perfection 4990 Photo) at 20 days after sowing. The images were analysed using a specifically-designed software program *Opengelphoto* (https://opengl.en.softonic.com/), which enables measurement of angle of individual roots from a vertical plane. For each seedling the growth angle between each of the first pair of seminal roots (i.e. left and right first pair of seminal roots) and the vertical plane was measured at approximately 3 cm distance from the seed (Figure 4, B). The SRN was measured by counting the number of roots based on the scanned images at the same date.

Statistical analysis

A linear mixed model framework was used to analyse genotype-by-environment interactions across experiments based on clear pots (Clear_1 and Clear_2) and growth pouches

(Pouch_1 and Pouch_2). The mixed model contained random components that identified the structure of the experimental design for each experiment: (i) Pot for the clear pot experiments, and (ii) Pouch and Box for the growth pouch experiment. Given the importance of genotype ranking across experiments, the random model formula also included Genotype as a random effect. The mixed model used for the clear pot experiments was:

$$y = \mu + g + p + e$$

where y is the response variable, μ is the general mean, g and p are random effects of genotype and pot respectively and e is the residual error. The mixed model used for the growth pouch experiment was:

$$y = \mu + g + b + b{:}p + e$$

where y is the response variable , μ is the general mean, g is the random effects of genotype, b is the random effect of box, $b{:}p$ is the random effect of pouch within box and e is the residual error. The random model formula allows for estimation of variance heterogeneity for each of the random terms for each experiment. The residual maximum likelihood algorithm (Patterson and Thompson, 1971) was used to provide estimates of the variance components and the best linear unbiased predictions (BLUPs). Data were analysed with ASReml-R (Butler *et al.*, 2009) using R software Version 3.0.0 (R Core team 2013).

For SRA measured using the growth pouch method, each plant had two values corresponding to the angle between the left or right seminal roots and the vertical plane. Therefore, the dataset for SRA measured using the growth pouch method had an additional factor Side (left and right). For SRA measured using the clear-pot method, each plant had a single value corresponding to the angle between the left and right seminal root. It could have been possible to measure the growth angle from the vertical plane as for the growth pouch method, but this would have generated more measurements resulting in decreased throughput. After the analysis, the BLUPs were multiplied by two to allow comparison with the seminal angle measured using the clear pot method. For SRN, a Student test was performed to compare the means between imaged and extracted SRN using R software Version 3.0.0.

Plant material

The study was conducted using a panel of 24 spring wheat cultivars (Table 2), that was previously characterized for SRA and SRN using a gel-filled chamber method reported by

Manschadi et al. (2008). In their study, Manschadi et al. (2008) obtained SRA ranging from 36° to 56° and SRN ranging from 3.2 to 5.0. These SRA values corresponded to the angle between each of the seminal roots and the vertical plane and were multiplied by two to allow comparison with the seminal angle measured in this study.

Table 2: Name, origin and genetic background of the 24 wheat cultivars used in this study

Cultivar	Breeding program[1]	Genetic background
Babax	CIMMYT	Veery
Baxter	QDAF	CIMMYT/Cook
Chara	DPI Vic	Cook/Pavon
Dharwar Dry	Central India	CIMMYT
Diamondbird	NSW DPI	Pavon
EGA Gregory	EGA	Pelsart/Batavia
EGA Hume	EGA	Pelsart/Batavia
EGA Wedgetail	EGA	Cook/Pavon
EGA Wentworth	EGA	Cook
Frame	AGT	Condor/Gabo
Giles	QDAF	Cook
Hartog	QDAF	Pavon
Janz	QDAF	Cook
Krichauff	AGT	Condor/Gabo
Lang	QDAF	Cook
Leichhardt	QDAF	Pavon
Petrie	QDAF	Pelsart/Batavia
SeriM82	CIMMYT	CIMMYT/Veery
Silverstar	NSW DPI	Cook/Pavon
Sunco	Uni Syd	Cook
Sunvale	Uni Syd	Cook
Ventura	NSW DPI	Cook/Pavon
Wyalkatchem	AgWA	Condor/Gabo
Yitpi	AGT	Condor/Gabo

[1] Breeding program abbreviations: Queensland Department of Agriculture and Fisheries (QDAF), Department of Primary Industries Victoria (DPI Vic), Australian Grain Technologies (AGT), New South Wales Department of Primary Industry (NSW DPI), International Maize and Wheat Improvement Center (CIMMYT), Enterprise Grains Australia (EGA), Western Australia Department of Agriculture (AgWA), University of Sydney (Uni Syd).

The panel comprised 21 Australian spring wheat cultivars, including some of the most widely grown throughout Australia in recent years, two elite cultivars (Babax and SeriM82) from the International Maize and Wheat Improvement Center (CIMMYT) in Mexico and one wheat cultivar from India (Dharwar dry).

Results

Genetic variation for seminal root traits

In the clear pots, seedling roots grew along the wall and were clearly distinguished from the dark soil. At the time of imaging for SRA (i.e. five days after sowing), the first pair of seminal roots had elongated on each side of the radicle, with an average SRA of 76° for the two clear pot experiments. By contrast, in the growth pouches, seedling roots grew freely in the air space between the moistened paper and the plastic. At the time of scanning (i.e. 20 days after sowing), first and often second pairs of seminal roots had elongated on each side of the radicle, however, only the angle between the first pair was considered here. The average SRA across the two pouch experiments was 110°. The observed range in SRA phenotypes varied between methods, with the clear pot method providing a range in SRA from 60 to 84° (i.e. a range of 24°), slightly wider range than the growth pouch method which produced a range from 101 to 117° (i.e. a range of 16°; Figure 5, A, and Appendix 1).

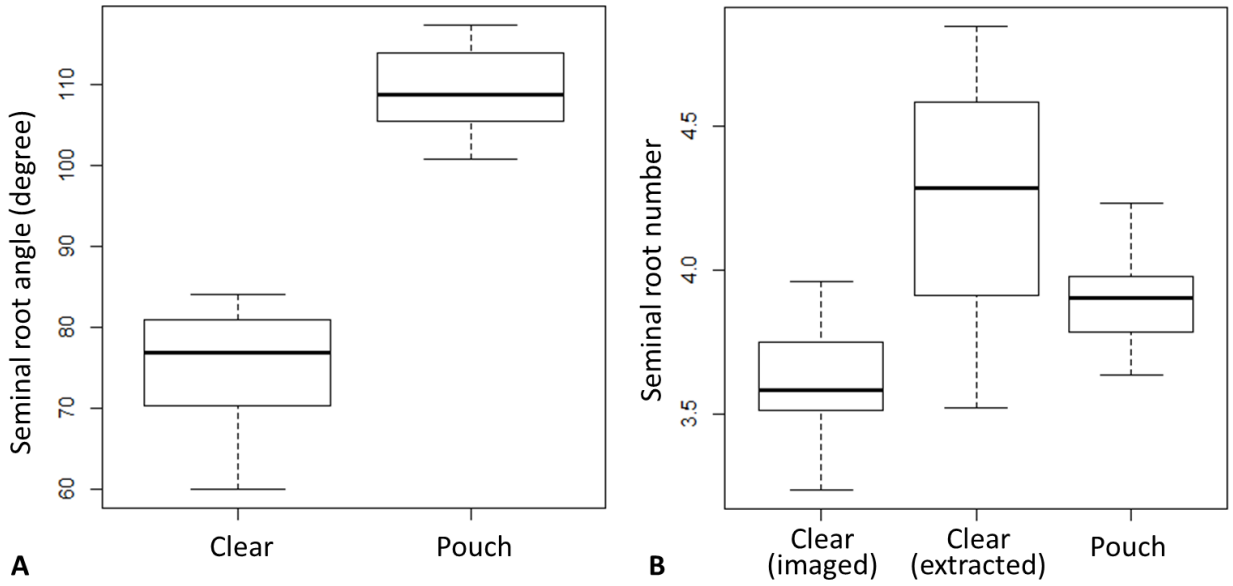

Figure 5: Genetic variation for seminal root traits

Box and whisker plots of (A) seminal root angle (SRA) and (B) seminal root number (SRN), for the panel of 24 wheat cultivars evaluated using the clear pot and growth pouch methods. The values correspond to the average BLUPs per cultivar of the two clear pot experiments Clear_1 and Clear_2 (Clear) and the two growth pouch experiments Pouch_1 and Pouch_2 (Pouch). The SRN for the clear pot method was measured either via image analysis (imaged) or by counting roots after removing seedlings from soil (extracted). The bottom and the top of the boxes display the first and third quartile values for each experiment, respectively. The band inside the box displays the median and the ends of the whiskers display the minimum and maximum values.

SRN was measured six days later than SRA in the clear pot experiments (i.e. at 11 days after sowing). In both clear pot experiments, SRN estimated non-destructively from the images was significantly lower (p-value < 0.001) compared to measures obtained by extracting the seedlings from the soil; average across the two experiments was 3.6 for imaged and 4.2 for extracted, respectively. In the pouch experiments, SRN was measured at the same time as SRA (i.e. at 20 days after sowing) and seedlings exhibited 3.9 roots on average across the experiments. The genotypic range in SRN phenotypes varied between methods, with the clear pot method providing the widest range in SRN (3.2–4.0 i.e. 0.8 for imaged and 3.5–4.8 i.e. 1.3 for extracted) compared to the growth pouch method (3.6–4.2 i.e. 0.6; Figure 5, B, and Appendix 1).

Evaluation of methods

The heritability for SRA was higher for the clear pot method (h^2 = 0.65) compared to the growth pouch method (h^2 = 0.52; Table 3). However, the heritability for each individual experiment displayed some variability within methods, with higher values for Clear_1 and Pouch_2 (h^2 = 0.79 and h^2 = 0.63, respectively) compared to Clear_2 and Pouch_1 (h^2 = 0.51 and h^2 = 0.42, respectively; Table 3). For SRN, the heritability was the highest for the clear pot method, with higher heritability obtained for extracted SRN (h^2 = 0.80) compared to imaged SRN (h^2 = 0.50; Table 3). The heritability for SRN was the lowest for the growth pouch method (h^2 = 0.37; Table 3). Overall, the heritability for each individual experiment was quite consistent within methods (Table 3).

Table 3: Statistics for seminal root traits

Heritability, genetic variance, error variance and average number of observations for seminal root angle (SRA) and seminal root number (SRN) for the panel of 24 wheat cultivars evaluated using different methods based on clear pots and growth pouches. The values correspond to the individual experiments. The values in bold correspond to the average of the two clear pot experiments Clear_1 and Clear_2 ('Clear average') and the two growth pouch experiments Pouch_1 and Pouch_2 ('Pouch average'). The SRN for the clear pot method was measured in two different ways: based on images (imaged) and after extracting the seedlings (extracted).

Trait	Experiment	Heritability (h^2)	Genetic variance	Error variance	Observations per cultivar
Seminal root angle	Clear_1	0.79	39%	61%	6.2/10
	Clear_2	0.51	16%	84%	5.7/10
	Clear average	**0.65**	**28%**	**72%**	**6.0/10**
	Pouch_1	0.42	6%	55%	4.5/6
	Pouch_2	0.63	14%	78%	5.3/6
	Pouch average	**0.52**	**10%**	**67%**	**4.9/6**

Seminal root number	Clear_1 (imaged)	0.45	9%	91%	8.2/10
	Clear_2 (imaged)	0.54	12%	86%	8.8/10
	Clear average (imaged)	**0.50**	**10%**	**90%**	**8.5/10**
	Clear_1 (extracted)	0.80	33%	66 %	8.2/10
	Clear_2 (extracted)	0.79	30%	69%	8.8/10
	Clear average (extracted)	**0.80**	**32%**	**68%**	**8.5/10**
	Pouch_1	0.37	9%	88%	5.2/6
	Pouch_2	0.36	8%	70%	5.7/6
	Pouch average	**0.37**	**9%**	**79%**	**5.5/6**

The error variance was higher than the genetic variance for all experiments (Table 3), indicating that there were more differences in the SRA and SRN between two plants of a genotype than between two plants of different genotypes. Almost all variation was explained by the genetic and error variance in the clear pot experiments. However, the random factors “Pouch” and “Box” had a significant effect in the growth pouch experiments.

The clear pot experiments (Clear_1 and Clear_2) used 10 reps per cultivar (i.e. 240 seeds in total per experiment), while the growth pouch experiments (Pouch_1 and Pouch_2) used only six reps per cultivar (i.e. 144 seeds in total per experiment). The number of observations for each experiment varied between experiments, as in both methods some seeds did not germinate and some roots were too short (< 3 cm) to measure SRA. Using the clear pot method, some roots were also hidden by the soil on the images, making measurement impossible. Roots were sometimes hidden by the soil close to the surface, but visible deeper down, making SRA measurement impossible but imaged SRN possible. In contrast, in the growth pouch method roots were always visible when present. The average number of observations per cultivar for SRA was 6.0 (out of 10) for the clear pot experiments and 4.9 (out of six) for the growth pouch experiments (Table 3). For SRN, the average number of observations per cultivar was 8.5 (out of 10) for the clear pot method for both imaged and extracted SRN while for the pouch method observations were obtained for 5.5 (out of six) plants per cultivar (Table 3).

The phenotypic correlations for the SRA were the highest between the two clear pot experiments Clear_1 and Clear_2 ($r^2 = 0.82$) and the lowest between the two growth pouch experiments Pouch_1 and Pouch_2 ($r^2 = 0.11$; Figure 6). The ranking of cultivars for SRA was almost the same across the two clear pot experiments, but differed markedly between the two growth pouch experiments. For instance, the cultivar Chara was the narrowest in

Pouch_1, but one of the widest in Pouch_2 (data not shown). The phenotypic correlation between the two methods, clear pot and growth pouch, were medium (r^2 ranging 0.37–0.48; Figure 6).

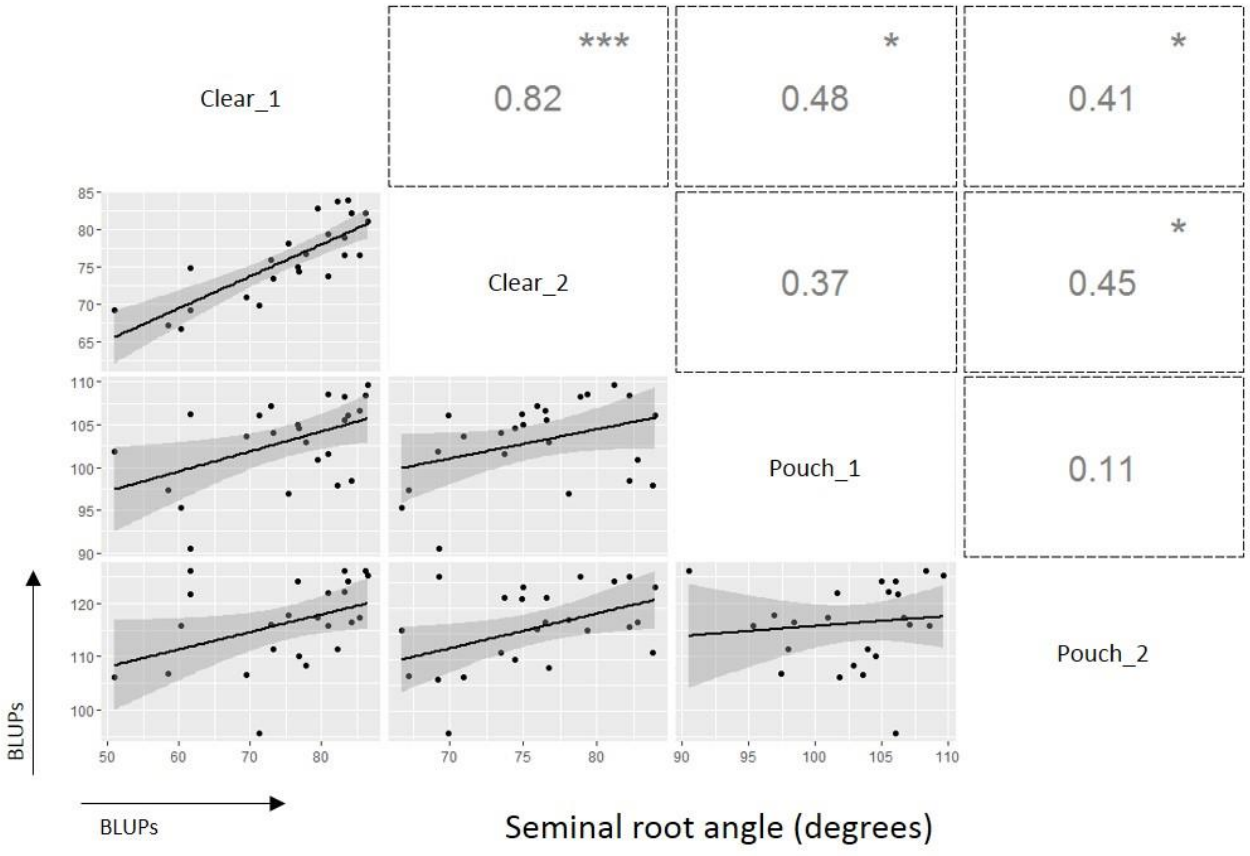

Figure 6: Phenotypic correlations of seminal root angle using clear pot and growth pouch methods
Phenotypic correlations (upper panels) and scatter plots (lower panels) of the BLUPs for seminal root angle (SRA, in degrees) between the clear pot (i.e. Clear_1 and Clear_2) and the growth pouch (i.e. Pouch_1 and Pouch_2) experiments. Data corresponds with average BLUPs of the 24 wheat cultivars. In the lower panels, the black line corresponds to a multiple linear regression line, with a linear smooth in dark grey. In the upper panels, the significance of the correlation is indicated with stars: *, P < 0.05; **, P < 0.01; ***, P < 0.001. No stars indicate that the correlation is not significant.

Phenotypic correlations between imaged and extracted SRN were high for both Clear_1 and Clear_2 experiments (r^2 = 0.85 and 0.75, respectively; Figure 7). The phenotypic correlations were high between the two clear pot experiments (Clear_1 and Clear_2) for the extracted SRN (r^2 = 0.63), but low for the imaged SRN (r^2 = 0.28; Figure 7). For the growth pouch method, the phenotypic correlation between the two experiments (Pouch_1 and Pouch_2) was medium (r^2 = 0.53), as well as the phenotypic correlations between clear pot (extracted) and growth pouch methods (r^2 ranging 0.37–0.64; Figure 7). There was no significant

phenotypic correlation between the SRA and SRN for either the clear pot or the growth pouch experiments (data not shown).

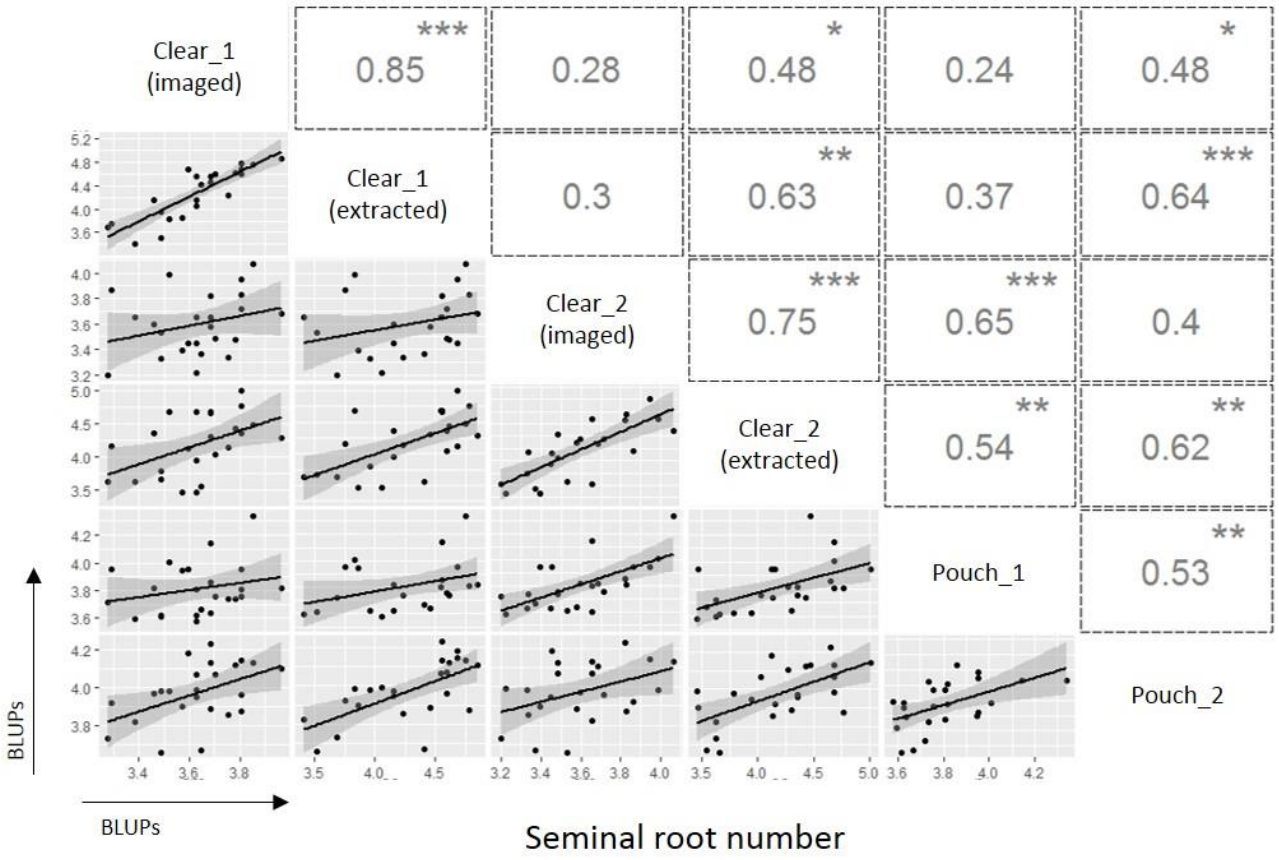

Figure 7: Phenotypic correlations of seminal root number using clear pot and growth pouch methods
Phenotypic correlations (upper panels) and scatter plots (lower panels) of the BLUPs for seminal root number (SRN) counted based on images (imaged) and after extracting the seedlings (extracted) for each of the clear pot experiments (i.e. Clear_1 and Clear_2), and for the SRN with the growth pouch experiments (Pouch_1, and Pouch_2). Data corresponds with average BLUPs of the 24 wheat cultivars. In the lower panels, the black line corresponds to a multiple linear regression line, with a linear smooth in dark grey. In the upper panels, the significance of the correlation is indicated with stars: *, P < 0.05; **, P < 0.01; ***, P < 0.001. No stars indicate that the correlation is not significant.

Diversity for seminal root angle in Australian wheat cultivars

The cultivar ranking for SRA was almost the same across the two clear pot experiments (Figure 8). Some trends based on genetic backgrounds could be observed, with all the Cook-type cultivars (EGA Wentworth, Giles, Janz, Lang, Sunco, and Sunvale) having narrower roots than all the Pavon-type cultivars (Diamonbird, Hartog, and Leichhardt) (Figure 8). Based on the pedigree (Table 2), the Cook/Pavon-type cultivars (Chara, EGA Edgetail, Silverstar, and Ventura) displayed a mixture of narrow and wide SRA phenotypes

as might be anticipated. Cultivars belonging to other genetic backgrounds did not show a consistent pattern of SRA.

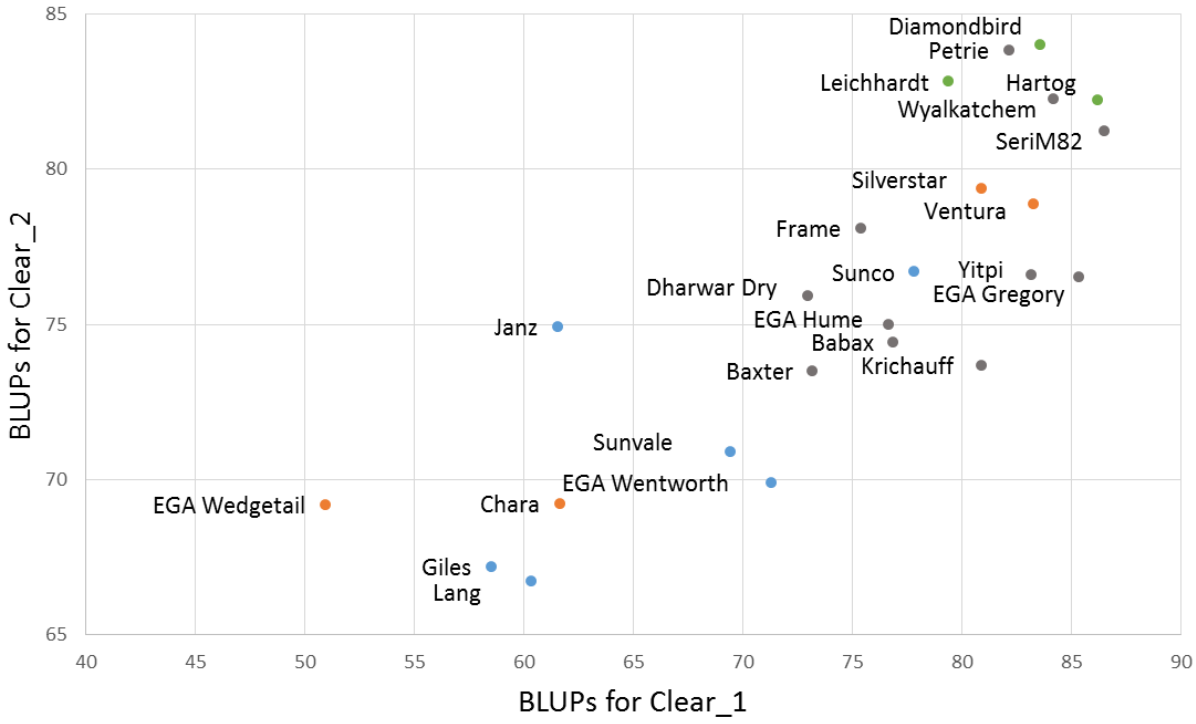

Figure 8: Seminal root angle of the panel of 24 wheat cultivars

Scatter plot of BLUPs for seminal root angle (SRA, in degrees) between the two clear pot experiments (i.e. Clear_1 and Clear_2) for 24 wheat cultivars. Blue dots = Cook-type, green dots = Pavon-type, orange dots =Cook/Pavon type, grey dots = other backgrounds.

Discussion

The two phenotypic methods for seminal root traits evaluated in this study permitted differentiation of SRA and SRN in the panel of 24 wheat cultivars. The newly developed method based on clear pots showed consistency across experiments, and is considered the most suitable for large-scale and high-throughput screening of seedling root characteristics in crop improvement programs.

Comparison of methods

In this study, we examined the SRA and SRN for a panel of 24 wheat cultivars measured using two methods; one based on clear pots and the other using growth pouches. The clear pot method provided a higher degree of variation for both seminal root traits with a range of 24° for SRA and 1.3 for extracted SRN. This compared to the growth pouch method with a

range of 16° and 0.6 roots per plant. It should be noted that these ranges may not represent the full extent of genetic variation in wheat germplasm, as this panel represents a limited set of genotypes and many share similar pedigrees and/or genetic backgrounds. Higher levels of variation for these traits were observed for the same 24 wheat cultivars in a previous study (72 – 112° i.e. 40º for SRA and 3.2 – 5.0 i.e. 1.8 for SRN) using a gel chamber method (Manschadi *et al.*, 2008). However, this method is more labour intensive and not suitable for evaluation of large numbers of entries. A difference of 34° was observed between average SRA provided by the clear pot method and the growth pouch method. Such difference could be attributed to the method itself. It is possible that air gaps between the paper wick and the plastic pouches may have constrained the roots to grow at wider angle than in the soil-filled clear pots. We believe that clear pots with soil are likely to more closely simulate the field situation than the paper wick of the plastic pouch system.

Despite variation within experiments, the heritability was higher using the clear pot method for both seminal root traits (i.e. $h^2 = 0.65$ for SRA and $h^2 = 0.80$ for extracted SRN) compared to the growth pouch method ($h^2 = 0.52$ for SRA and $h^2 = 0.37$ for SRN). If implemented in breeding programs, the relatively high heritability should enable genetic gain for these traits. The achieved number of observations for SRA using the clear pot method was lower than the potential 10 observations due to the fact that some roots were hidden by soil in the images. As a consequence, this method requires a high number of repetitions (i.e. ~10) to ensure high heritability. The position of the seed at sowing (i.e. embryo pointed downwards and slightly towards the wall) is critical to ensure roots grow along the wall and are visible. The achieved number of observations for seminal root traits using the growth pouch method was close to the potential six observations due to the fact that roots were always visible when present. The heritability could be improved by increasing the number of reps, for example 10 reps instead of six. The error variance was higher than the genetic variance for all experiments, which is not surprising considering that traits were measured for single plants. Results from the two clear pot experiments were more strongly correlated ($r^2 = 0.82$ for SRA and $r^2 = 0.75$ for extracted SRN), when compared to results from the two growth pouch experiments ($r^2 = 0.11$ for SRA and $r^2 = 0.53$ for SRN). The rank of the cultivars based on SRA and SRN was quite consistent across the two clear pot experiments, suggesting that the method is repeatable and has power to detect differences in root phenotypes (i.e. narrow/wide SRA, low/high SRN). The wider range of root trait values observed for phenotypes using the clear pot method enabled better differentiation among cultivars with

more repeatable results, and thus appears superior to the growth pouch method for implementation in breeding programs.

SRN was measured with the clear pot method in two different ways: by counting based on images and after seedlings were extracted from the soil. Roots were underestimated using images because some roots were hidden by soil, resulting in a significantly lower average SRN for imaged SRN compared to extracted SRN. As expected, extracted SRN was more accurate than imaged SRN. For instance, genetic variation, heritability and phenotypic correlations were higher for extracted SRN than imaged SRN. However, imaged and extracted SRN were strongly correlated ($r^2 > 0.75$) and ranking of cultivars using both techniques was also very similar. Despite a lower level of precision, estimation of SRN using the imaging technique is preferred for breeding purposes because this method doesn't require a labour intensive transplanting of the selected plants. For instance, the imaging method can be used to differentiate extreme phenotypes (i.e. low versus high SRN), in order to enrich segregating populations with desirable genes via repetitive cycles of selection or discard undesired phenotypes. However, to precisely phenotype or characterize fixed lines, counting the roots after pulling out the plants may be preferred.

The paper growth medium in growth pouches and the agar gel of the gel-filled chamber method from Manschadi et al. (2006; 2008) both provide conditions less representative of natural soils than the soil-based growth medium used in the clear post system. Consequently the soil-based clear pot method may result in phenotypes more similar to those expressed in the field (Gregory *et al.*, 2009). In addition, the growth pouch and gel-filled methods are very time-consuming and labour intensive to set up, thus, are better suited for evaluation of smaller numbers of genotypes compared to the clear pot method. For these reasons, we propose that the clear pot method is preferred for high-throughput and large-scale screening of SRA and SRN.

Opportunities for plant breeding

The rank between cultivars based on the SRA calculated with the clear pot method was almost identical across the two experiments and ranking seemed to correspond with the genetic background of the wheat cultivars. For instance, most of the Cook-type cultivars displayed a narrow SRA, while all the Pavon-type cultivars displayed wider SRA, which is similar to previous studies (Manschadi *et al.*, 2008; Christopher *et al.*, 2013). The Cook-type cultivars tend to have a longer season maturity compared to the Pavon-type cultivars used

in this study. Cultivars with a longer cycle are more likely to encounter terminal moisture stress in the season, particularly if grown in a summer dominant rainfall environment. Deeper rooting could be an adaptation for late cultivars to ensure photosynthetic and remobilization activities during grain filling in rain-fed wheat production systems relying heavily on deep stored soil moisture. However, considering the small sample set and other confounding factors, field studies are required to confirm this hypothesis. There was little consistency between the preferred growing region for the Australian wheat cultivars evaluated in this study and cluster analysis based on SRA phenotypes also failed to detect any obvious trends other than those associated with genetic background (data not presented). Although wheat breeders have likely indirectly selected for desirable root architecture where environmental pressure is frequent, this is not the only trait affecting drought adaptation. In fact, while drought types differ greatly depending on the season and region (Chenu *et al.*, 2011, 2013; Chenu, 2014), drought adaptation typically involves the interaction of a number of traits related to water utilisation as well with other physiological processes (Slafer, 2003; Fischer and Edmeades, 2010). As a result, breeders and pre-breeders are targeting other traits such as adapted phenology (Gomez-Macpherson and Richards, 1995), transpiration efficiency (Rebetzke *et al.*, 2013), cooler canopy temperature (Blum *et al.*, 1989; Olivares-Villegas *et al.*, 2007; Rebetzke *et al.*, 2012*b*) and reduced tillering (Mitchell *et al.*, 2012). While deep root architecture is likely important for adaptation in rainfed wheat production systems relying heavily on stored soil moisture (particularly at depth, Manschadi *et al.*, 2006), this trait may be less advantageous in other environments, for example where rainfall is more frequent through the growing season, where soils are compacted (Rich and Watt, 2013) or for late sown conditions (Saxena *et al.*, 2014).

Selection for combinations of physiological traits that underpin yield may be a more effective way to achieve genetic gain for yield in specific environment types, rather than direct selection for yield *per se* (Jackson *et al.*, 1996; Chapman *et al.*, 2003; Hammer *et al.*, 2005; Chenu, 2014). The clear pot method allows high-throughput and cost-effective screening of breeding populations at a rate of 600 plants.m^{-2} in controlled environment conditions within only five days for SRA and 11 days for SRN. The technique is suitable for characterising both fixed lines and for screening large segregating populations. As the system permits growing-on of the selected plants, repeated cycles of selection can be performed across consecutive generations to rapidly enrich breeding populations with desirable alleles for root traits. Alternatively, the method could be used to select parental lines with desired root traits

for crossing. Therefore, the clear pot method has the potential to accelerate genetic gain for drought adaptation in breeding programs.

The technique is also well adapted for use in the 'speed breeding' system developed and refined at The University of Queensland that achieves rapid plant growth by incorporating controlled temperature and constant light (O'Connor *et al.*, 2013). By combining speed-breeding growth conditions and the root trait phenotypic screening method, it is possible to achieve up to 30 phenotypic screens within 12 months if plants are not grown to maturity. Alternatively, under optimised growth conditions, up to six consecutive cycles of selection could be achieved in 12 months with selections grown through to maturity producing seed in each generation. Thus, within a 12 month timeframe, it would be possible to make crosses, screen and produce seeds for F_1 to F_4 generations for desirable root traits, and produce $F_{5:6}$ lines with improved root traits. Also, seminal root trait screening can be easily integrated with other phenotypic screening methods adapted to the speed breeding system, such as adult plant resistance to rust pathogens (Hickey *et al.*, 2011) and grain dormancy for adaptation to pre-harvest sprouting (Hickey *et al.*, 2010). The clear-pot method has been successfully used in barley (*Hordeum vulgare* L.), to identify genomic regions influencing seminal root traits (Robinson *et al.*, 2016). We anticipate this methodology could be applied to wheat, and to other crops, such as rice (*Oryza sativa* L.) and chickpea (*Cicer arietinum* L.).

Conclusions

Phenotyping root traits in wheat has been limited by the availability of suitable methods. In this chapter, we report a novel high-throughput method using clear pots to phenotype root architectural traits. For the first time, seminal root traits such as SRA can be easily measured in 5-day-old wheat seedlings and SRN in 11-day-old wheat seedlings. This method has clear advantages over other previously reported techniques and could be easily integrated into wheat breeding programs targeting drought adaptation via improved plant access to deep soil water.

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Chapter 4
Rapid introgression of desirable alleles for seminal root angle in wheat

Abstract

In Chapter 3, we developed a method to rapidly phenotype proxy traits for root system architecture in large populations of wheat (*Triticum aestivum* L.). We suggested this method could be applied in breeding programs to select for desirable root traits. In this chapter, we studied the potential to apply the method described in Chapter 3 to manipulate seminal root angle in early generations of wheat via direct phenotypic or molecular selection. Using the clear-pot method, we characterised a panel of 22 wheat lines adapted to Australian environments. We selected parental lines having contrasting phenotypes, and developed three backcross populations of interest to breeders. To test the ability to rapidly shift population distribution and allele frequency, we applied selection in segregating generations (BC_1F_2 and BC_1F_3) to develop tail populations for both 'narrow' and 'wide' root angle. Overall, two consecutive rounds of selection significantly shifted the mean root angle by up to 10°. Further characterisation of 46 fixed lines from a tail population selected for narrow root angle, identified ten lines with root angle phenotypes significantly narrower than the recurrent parent. Allele frequency comparison between 'narrow' and 'wide' tails revealed genetic regions under selection. Marker-assisted selection for these regions successfully identified five 'narrow' and five 'wide' lines in an independent population derived from the same parental lines. These results suggest that there is a valuable source of allelic variation for root angle that can be harnessed and introgressed into elite wheat lines to improve sustainable wheat production in terminal drought environments.

Introduction

A number of drought-adaptive shoot traits have been integrated into crop breeding programs to accelerate the development of high-yielding cultivars (Sinclair *et al.*, 2004; Richards, 2006). However, few successful examples have been reported for the 'hidden half'. Plant breeders are limited in their ability to select for below-ground traits. Roots are difficult to phenotype, and exhibit complex environmental and genetic controls (Passioura, 1983; Yu

et al., 2006; Bengough *et al.*, 2006; Ito *et al.*, 2006; Lynch, 2007). Nonetheless, there is some indication that aspects of the root system may have been subject to indirect selection in crop breeding programs (de Dorlodot *et al.*, 2007; Hammer *et al.*, 2009). Hence, demonstrating that desirable root architectural traits can be rapidly introgressed into elite lines of wheat (*Triticum aestivum* L.) would provide evidence to breeders that such strategies could be worth pursuing.

Previous studies indicate that root architectural traits expressed at earlier stages are associated with improved yield in water-limited environments in rice *(Oryza sativa* L., Uga *et al.*, 2013), sorghum (*Sorghum bicolor* L., Mace *et al.*, 2012), and wheat (Manschadi *et al.*, 2006). One such trait is seminal root angle (SRA, Manschadi *et al.*, 2006; Kato *et al.*, 2006; Wasson *et al.*, 2012). As described in the previous chapter, SRA is highly heritable and can be rapidly screened at early developmental stages in wheat seedlings using clear pots. Narrow SRA has been proposed as a secondary selection criterion in wheat breeding programs to target improved water use at depth and adaptation to target cropping environments (Manschadi *et al.*, 2010; Wasson *et al.*, 2012; Richard *et al.*, 2015; Casadebaig *et al.*, 2016). Incorporation of this proxy trait into breeding programs would accelerate the deployment of favourable root system architecture genes in elite wheat lines.

In this chapter, we investigated the effectiveness of direct phenotypic selection and marker-assisted selection (MAS) for SRA. We used the clear-pot method developed in Chapter 3 to rapidly apply two rounds of bi-directional selection for SRA in early generations (BC_1F_2 and BC_1F_3) of three backcross populations. We examined shifts in population distribution resulting from the two selection cycles, and characterised fixed lines ($BC_1F_{4:5}$) generated in one of the tail populations selected for narrow SRA. We investigated shifts in allelic frequency by comparing allele frequency of the recurrent parent in the tails, and identify regions under selection. We also tested the effectiveness of MAS for these regions in an independent $F_{4:5}$ population. We discuss the opportunities and potential limitations of this method to integrate effective selection for SRA into breeding programs.

Materials and methods

Plant material

A panel of 22 candidate parental wheat lines, comprising cultivars and elite breeding lines adapted or with potential adaptation to the Australian cropping conditions, was assembled.

This panel was used to identify six parental lines for developing three backcross populations of interest to breeders. The panel includes cultivars and elite breeding lines from Australian breeding programs, an Indian cultivar, lines from the International Center for Agricultural Research in the Dry Areas (ICARDA) and the International Maize and Wheat Improvement Center (CIMMYT; Table 4). Notably, in this panel, some lines share common genetic background. For example, Mace, UQ01687, and Wallup are derived from Wyalkatchem; RIL114 and UQ01648 from H45; and Spitfire from Drysdale (Table 4).

Table 4: Details of the 22 candidate parental wheat lines comprising the panel used in this study

Name	Type	Breeder[1]	Pedigree
36:ZWW11	Elite breeding line	CIMMYT	EGA Bonnie Rock/4/Milan/Kauz//Prinia/3/BAV92
8:ZWW11	Elite breeding line	CIMMYT	D67.2/P66.270//AE.Squarrosa (320)/3/Cunningham/4/Vorb
Dharwar Dry*	Cultivar	India	DWR39/C306//HD2189
Drysdale	Cultivar	CSIRO	Hartog*3/Quarrion
EGA Gregory*	Cultivar	EGA	Pelsart/2*Batavia
FAC10-16	Elite breeding line	ICARDA	10CB-F/W234
Hartog*	Cultivar	QDAF	Vicam 71//Ciano 's'/Siete Cerros/3/Kalyansona/Bluebird
Mace	Cultivar	AGT	Wyalkatchem/Stylet//Wyalkatchem
QT14617	Elite breeding line	QDAF	CMSS96M00584S-050M-040Y-0100M-020Y-31M-0Y
RIL114	Elite breeding line	UQ	UQ01484/RSY10//H45
SB062	Elite breeding line	CIMMYT	Seri M82/Babax
Scout	Cultivar	LPB	Sunstate/QH71-6//Yitpi
SeriM82*	Elite breeding line	CIMMYT	Kavkaz/4/Saric F 70///Lerma Rojo 64A/Inia F66//Inia F66/Yecora F70/5/II-26992
Spitfire	Cultivar	LPB	Drysdale/Kukri
Suntop	Cultivar	AGT	Sunco/2*Pastor//SUN436E
UQ01648	Elite breeding line	UQ	UQ01484/RSY10//2*H45
UQ01687	Elite breeding line	UQ	UQ01484/RSY10//2*Wyalkatchem
Wallup	Cultivar	AGT	Wyalkatchem/Chara
Westonia	Cultivar	Intergrain	Spica/Timgalen//Tosca/5/Wren:Mex//Ciano F 67/Noroeste F 66///Zambezi/4/Jacup*2/Bobwhite
ZWB10-37	Elite breeding line	CIMMYT	Tacupeto F2001/Brambling//Kiritati
ZWW10-128	Elite breeding line	CIMMYT	ESDA/KKTS
ZWW10-50	Elite breeding line	CIMMYT	Onix/4/Milan/Kauz//Prinia/3/BAV92

[1] Breeding program abbreviations: Australian Grain Technologies (AGT), International Maize and Wheat Improvement Center (CIMMYT), Commonwealth Scientific and Industrial Research Organisation (CSIRO), Enterprise Grains Australia (EGA), International Center for Agricultural Research in the Dry Areas (ICARDA), LongReach Plant Breeders (LPB), Queensland Department of Agriculture and Fisheries (QDAF), The University of Queensland (UQ)

* These lines were shared with Chapter 3, table 2

Selection of parental lines

The panel of 22 candidate parental wheat lines was characterised for SRA using the clear-pot method described in Chapter 3 in two repeated experiments: CandP–1 and CandP–2. The two experiments used a randomized complete block design, where ten seeds of each of the 22 lines were randomised across ten pots. Data from repeated characterisation of the panel was analysed using a mixed model, containing 'Line' (i.e. cultivars or breeding lines) and 'Rep' as random components. Best linear unbiased predictions (BLUPs) were obtained for each line in each experiment (CandP–1 and CandP–2) using ASReml-R (Butler *et al.*, 2009) and R software Version 3.2.0 (R Core team 2013).

Six parental lines having different phenotypes for SRA were selected from the panel and used to develop three backcross populations segregating for the trait of interest. These three backcross populations used three Australian spring wheat cultivars as recurrent parents and three diverse donor parents. The recurrent parents, namely Mace, Scout, and Suntop, are widely grown throughout the western, southern, and eastern regions of the Australian wheat belt, respectively. The donor parents, namely Dharwar Dry, Drysdale, and SB062, have desirable traits for drought and/or heat adaptation. For instance, Dharwar Dry is adapted to rain-fed wheat production in India, with a deep root system and stay-green phenotype (Manske and Vlek, 2002; Manschadi *et al.*, 2008). Drysdale is known for superior transpiration efficiency (Condon *et al.*, 2004; Tausz-Posch *et al.*, 2012), and SB062 is a breeding line from CIMMYT, with tolerance to warm conditions (Chenu et *al*. unpublished) with low canopy temperature and high levels of water soluble carbohydrates (Olivares-Villegas *et al.*, 2007; Dreccer *et al.*, 2009).

Two populations with parents having contrasting phenotypes for SRA (i.e. narrow SRA for donor parents versus wide SRA for recurrent parents) were developed, namely Pop1 – Ma/Dr (Mace/Drysdale//Mace)[1] and Pop2 – Su/Dh (Suntop/Dharwar Dry//Suntop)[1]. One population with parents having intermediate SRA phenotypes was further developed, namely Pop3 – Sc/SB (Scout/SB062//Scout)[1]. Pop1 – Ma/Dr and Pop2 – Su/Dh were designed to test the ability to introgress narrow SRA into elite cultivars exhibiting wide SRA, whereas Pop3 – Sc/SB provided an opportunity to explore the ability to manipulate SRA in crosses derived from parents exhibiting similar SRA phenotypes. Despite having a similar SRA phenotype, Scout and SB062 are genetically distant. These parental lines have a

[1] (Parental Line / Parental Line // Recurrent Parent)

coefficient of parentage of 0.19, based on a calculation from Kempthorne (1969) using the assumptions that each parent contributes equally and that ancestors without known pedigrees are unrelated. As SRA is under complex genetic control and determined by a combination of alleles with both positive and negative effects (Christopher *et al.*, 2013), lines displaying similar phenotypes could have different combinations of alleles.

Growing conditions (speed breeding system)

All generations (except F_2 for Pop1 – Ma/Dr) were grown in the speed breeding system, with controlled temperature (22 ± 3°C) and constant (24 h) light (O'Connor *et al.*, 2013). At ten days after sowing, slow release Osmocote® NPK fertiliser (N – P – K: 21.2 – 1.9 – 5.7, with trace elements) was supplied (5 g L^{-1}). Using this system, lines developed to the $BC_1F_{4:5}$ or $F_{4:5}$ generation in this study were obtained within 18 months.

For generations that were subject to phenotyping, seedlings were grown for five days from sowing in a climate-controlled growth facility with environmental conditions maintained at 12 h photoperiod and constant temperature of 17°C, as required for SRA assessment (Chapter 3).

Development of tail backcross BC_1F_4 populations and cycles of phenotypic selection

Three F_1 crosses were made (Mace/Drysdale, Suntop/Dharwar Dry and Scout/SB062) and backcrossed to Mace, Suntop, and Scout, respectively. The resulting BC_1F_1 seeds were bulked for each backcross population and grown in the glasshouse to produce BC_1F_2 seeds for the three backcross populations Mace/Drysdale//Mace (Pop1 – Ma/Dr), Suntop/Dharwar Dry//Suntop (Pop2 – Su/Dh), and Scout/SB062//Scout (Pop3 – Sc/SB), respectively. The three backcross populations, Pop1 – Ma/Dr, Pop2 – Su/Dh, and Pop3 – Sc/SB were developed to the BC_1F_4 generation, by combining generations of single seed descent with phenotyping selection for SRA (Figure 9). As Pop1 – Ma/Dr was observed to be clearly segregating for the trait of interest, the 'narrow' tail of Pop1 – Ma/Dr was progressed an additional generation to obtain $BC_1F_{4:5}$ plants (Figure 9).

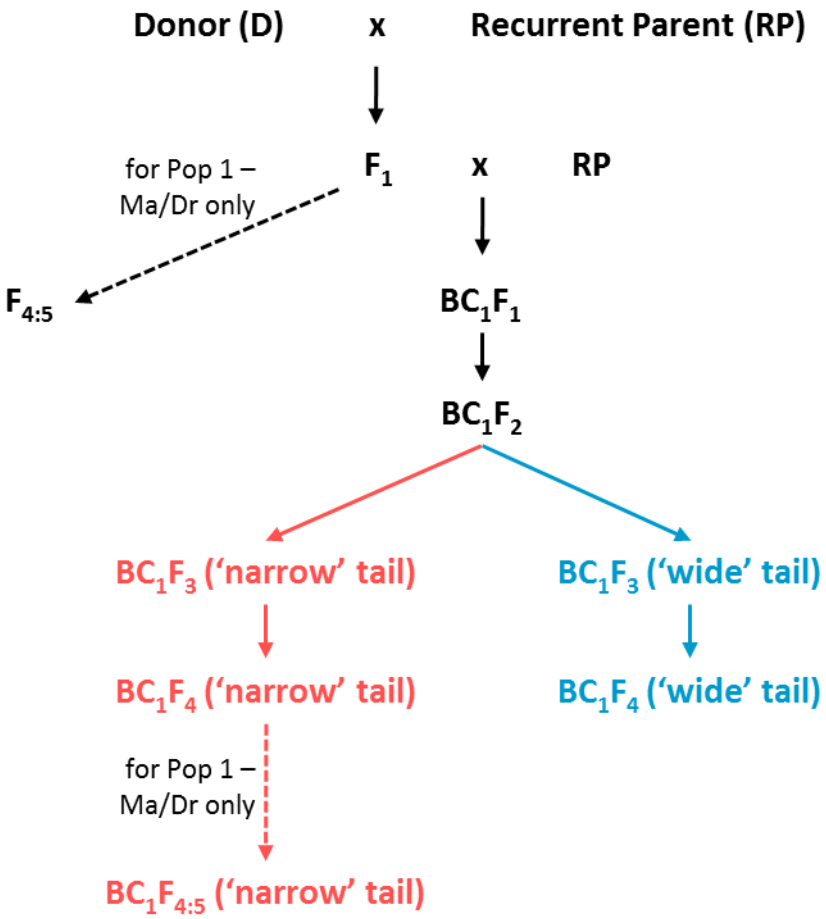

Figure 9: Scheme for developing tail backcross populations for seminal root angle and an independent population

This scheme was applied to develop the 'narrow' and 'wide' tail populations up to the BC_1F_4 generation (represented in pink and blue respectively), for the three populations: Pop1 – Ma/Dr (Mace/Drysdale//Mace), Pop2 – Su/Dh (Suntop/Dharwar Dry//Suntop) and Pop3 – Sc/SB (Scout/SB062//Scout). The 'narrow' tail population for Pop1 – Ma/Dr was progressed an additional generation to the $BC_1F_{4:5}$ generation (represented by the pink dashed line). The F_1 generation from Pop1 – Ma/Dr was independently progressed to the $F_{4:5}$ generation through selfing (represented by the black dashed line). No selection was applied in this independent population. RP: Recurrent parent (i.e. Drysdale, Dharwar Dry and SB062 for Pop1 – Ma/Dr, Pop2 – Su/Dh and Pop3 – Sc/SB, respectively); D: Donor (i.e. Mace, Suntop and Scout for Pop1 – Ma/Dr, Pop2 – Su/Dh and Pop3 – Sc/SB, respectively).

Two rounds of bi-directional selection for SRA were applied to segregating generations (BC_1F_2 and BC_1F_3) of the three populations. SRA was assessed using the clear-pot method as described in Chapter 3. The number of plants assessed for SRA varied between populations and generations of screening (Table 5). This was because some roots were hidden by the soil, while others were too short (<3 cm) at the time of imaging, or seeds did not germinate. Thus, to maintain the population size throughout the development of the backcross populations, different selection intensities were applied for each population and generation (Table 5).

Table 5: Characteristics of the selection phases for developing the tail populations

Selection phases are presented at each generation, for each tail population selected for narrow or wide seminal root angle (SRA), of three backcross populations, Pop1 – Ma/Dr (Mace/Drysdale//Mace), Pop2 – Su/Dh (Suntop/Dharwar Dry//Suntop), and Pop3 – Sc/SB (Scout/SB062//Scout).

Population	Tail	Generation	Sown plants	Assessed plants[1]	Selected plants[2]	Selection intensity[3]	BC_1F_2 families[4]
Pop1 – Ma/Dr	Narrow	BC_1F_2	552	292	60	21%	-
		BC_1F_3	276	136	37	27%	25
		BC_1F_4	276	193	46 (49)	24% (25%)	20
	Wide	BC_1F_2	552	292	60	21%	-
		BC_1F_3	276	130	37	29%	27
		BC_1F_4	276	179	- (46)	- (26%)	-
Pop2 – Su/Dh	Narrow	BC_1F_2	552	245	57	23%	-
		BC_1F_3	276	151	41	27%	28
		BC_1F_4	276	174	-	-	-
	Wide	BC_1F_2	552	245	58	24%	
		BC_1F_3	276	154	41	27%	28
		BC_1F_4	276	177	-	-	-
Pop3 – Sc/SB		BC_1F_2	552	286	60	21%	-
	Narrow	BC_1F_3	276	111	32	29%	24
		BC_1F_4	276	125	- (34)	- (27%)	-
	Wide	BC_1F_2	552	286	60	21%	-
		BC_1F_3	276	102	32	31%	24
		BC_1F_4	276	143	- (35)	- (24%)	-

[1] The number of plants for which the seminal root angle could unambiguously be measured.

[2] The number of plants from which seeds were used in the next generation for SRA assessment. The numbers in brackets indicate the number of plants selected for genotyping at the BC_1F_4 generation. '-' indicates that the population was not progressed past this generation.

[3] The proportion of plants selected for SRA assessment at each generation. The proportions in brackets indicate the proportion of plants selected for genotyping at the BC_1F_4 generation. '-' indicates that the population was not progressed past this generation.

[4] The number of BC_1F_2 families from which the BC_1F_3 and BC_1F_4 were selected, are presented as an indicator of the selection pressure relative to the first selection step as not all families selected in the BC_1F_2 were necessarily carried forward in subsequent generations. '-' indicates that the population was not progressed past this generation.

For screening the BC_1F_2 generation, 552 seeds per population were sown in clear pots. The experiment was blocked according to each of the three populations and 12 replicates for each of the respective parents were included in each block. Each block contained 24 pots, where each pot contained 23 progeny plus one parent line randomly allocated to a position. At five days after sowing, SRA was determined for each individual plant via image analysis. Each BC_1F_2 plant screened for SRA was considered an individual genotype, thus no replication was possible and raw values were used to generate population distributions. Within each population, individuals displaying extreme phenotypes, thus representing both

the lower ('narrow' angle) and upper ('wide' angle) tails of the population distribution were selected (respectively represented by pink and blue shaded areas in Figure 10). Selection intensity ranged from 21 to 24%, resulting in tail populations comprising 57 to 60 BC_1F_2 plants (Table 5). The selected plants were grown-on to produce self-pollinated seeds (BC_1F_3), while the non-selected plants were discarded. The BC_1F_3 seeds were harvested on a per plant basis (i.e. per BC_1F_2 family). In total, six separate tail populations were created: 'narrow' and 'wide' SRA for each of the three main populations. Four to five BC_1F_3 seeds from the selected BC_1F_2 plants were sampled, bulked, and grown to be screened again and provide the next generation.

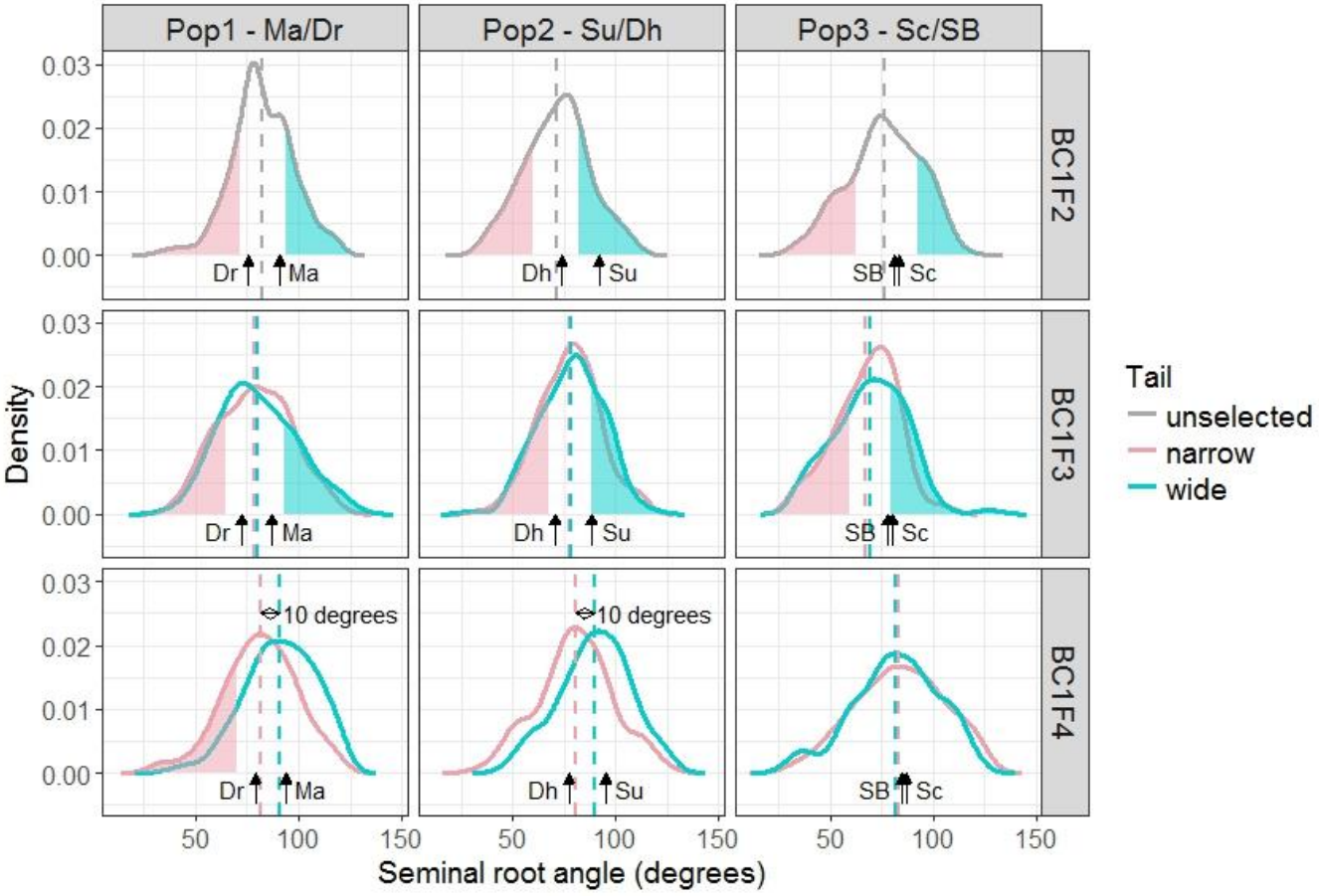

Figure 10: Distribution of seminal root angle for each generation and each population

The frequency distribution of seminal root angle (SRA) is presented for individuals from the BC_1F_2, BC_1F_3 and BC_1F_4 generations for each of the three populations: Pop1 – Ma/Dr (Mace/Drysdale//Mace), Pop2 – Su/Dh (Suntop/Dharwar Dry//Suntop) and Pop3 – Sc/SB (Scout/SB062//Scout). The shaded portion of the distribution indicates the selected individuals retained following bi-directional selection in each generation, where pink shading indicates the 'narrow' tail and blue shading indicates the 'wide' tail. The grey dashed line represents the mean SRA attained by the BC_1F_2 population, while pink and blue dashed lines display the mean SRA for the BC_1F_3 and BC_1F_4 plants from the 'narrow' and 'wide' tail populations, respectively. Arrows display SRA for donor lines Drysdale (Dr), Dharwar Dry (Dh), and SB062 (SB), and respective recurrent parents Mace (Ma), Suntop (Su), and Scout (Sc), for each population in each experiment.

For screening the BC_1F_3 generation, a total of 276 seeds for each of the six tail populations were sown in clear pots and assessed for SRA. Again, the experiment was blocked according to population and replicates for each of the respective parents were included, as described above. Similarly, each BC_1F_3 plant screened for SRA was considered an individual genotype and raw values were used to generate population distributions. Within 'narrow' tail populations, individuals exhibiting extreme narrow phenotypes were again selected, while within the 'wide' tail populations, plants displaying extreme wide phenotypes were again selected (Figure 10). Within each tail, selection intensity ranged from 27 to 31%, resulting in tail populations of 32 to 41 BC_1F_3 plants. Sometimes, no individuals from a BC_1F_2 family were retained. Thus, not all families selected in the BC_1F_2 generation were necessarily carried forward in subsequent generations. Here, selected BC_1F_3 plants were sampled from 24 to 28 BC_1F_2 families (Table 5). The selected BC_1F_3 plants were retained and grown-on in the glasshouse to produce BC_1F_4 seeds, while the non-selected plants were discarded. The BC_1F_4 seeds were harvested on a per plant basis. Six to nine BC_1F_4 seeds were sampled per selected BC_1F_3 plant, bulked, and grown on to be phenotyped.

For screening the BC_1F_4 generation, a total of 276 seeds for each of the six tail populations were sown in clear pots and assessed for SRA. The experiment was conducted as described above for screening the BC_1F_3 generation. To compare shifts in population distribution over the course of line development, a Welch two sample t-test was used to compare SRA means attained by 'narrow' and 'wide' tail populations for each backcross population in the BC_1F_3 and BC_1F_4 generations.

Development and characterisation of $BC_1F_{4:5}$ lines

Pop1 – Ma/Dr, which was visibly segregating for SRA, was progressed an additional generation to obtain $BC_1F_{4:5}$ plants (Figure 9 and Figure 10). A selection intensity of 24% was applied to select the lower tail of the SRA distribution, resulting in the selection of 46 BC_1F_4 plants, derived from 20 BC_1F_2 families (Table 5) and 26 BC_1F_3 families. The selected 46 BC_1F_4 plants from Pop1 – Ma/Dr were grown-on in the glasshouse to produce $BC_1F_{4:5}$ seeds, which were considered fixed lines.

The 46 $BC_1F_{4:5}$ lines from the 'narrow' tail of Pop1 – Ma/Dr were characterised for SRA, along with associated parental lines Drysdale and Mace using the clear-pot method described in Chapter 3. As the $BC_1F_{4:5}$ lines were considered fixed, a randomized complete block design was employed, where ten replicate seeds of each of the 46 lines along with the

two parental lines were randomized across 20 pots. SRA was analysed using a mixed model, containing 'Line' and 'Rep' as random components. BLUPs were obtained for each line as described above, and grouped according to a Fisher's LSD test (p-value = 0.05) using the package ASRemlPlus.

Genotyping and comparative marker allele frequency analysis of BC_1F_4 lines

Genomic DNA was extracted from young leaf tissue using the CTAB-based extraction protocol recommended by Diversity Arrays Technology Pty Ltd (DArT; www.diversityarrays.com). The samples submitted to DArT for genotyping consisted of selected individuals from the 'narrow' and 'wide' tail populations of Pop1 – Ma/Dr and Pop3 – Sc/SB, as well as respective parental lines. Individuals exhibiting extreme narrow phenotypes within 'narrow' tail populations and individuals displaying extreme wide phenotypes within the 'wide' tail populations of Pop1 – Ma/Dr and Pop3 – Sc/SB were selected with selection intensity ranging from 24 to 27% (Table 5). In total, 49 and 46 BC_1F_4 lines from the 'narrow' and 'wide' tail population of Pop1 – Ma/Dr, 34 and 35 BC_1F_4 lines from the 'narrow' and 'wide' tail population of Pop3 – Sc/SB , and one sample of each of the parental lines (Drysdale, Mace, SB062, and Scout), were genotyped using the wheat genotype by sequencing platform. Genotyping returned scores for dominant markers extracted *in silico* from sequences obtained from genomic representations referred to as SilicoDArT markers. Here, 4,827 and 2,640 polymorphic SilicoDArT presence-absence markers were returned for Pop1 – Ma/Dr and Pop3 – Sc/SB, respectively. SilicoDArT markers were positioned on the wheat DArT consensus map provided by Dr Andrzej Killian from DArT.

Marker data was processed using a quantitative allele frequency analysis method, referred to as comparative marker frequency analysis (Ziems *et al.*, 2017). For both Pop1 – Ma/Dr and Pop3 – Sc/SB, frequencies of the recurrent parent allele in the 'narrow' and 'wide' tail populations were compared in the BC_1F_4 progeny. For each marker, a discriminant value reflecting the difference in allele frequency between the two groups was calculated (Wenzl *et al*., 2006, 2007). This method identifies genetic loci conditioning phenotypic characteristics with at least 5-centimorgan (cM) accuracy without the requirement of a linkage map (Wenzl *et al*., 2007). A Chi-squared test was performed at each marker to detect significant discrimination between the expected and observed allele frequencies. A differential threshold of > 0.4 discriminant value and false discovery rate adjusted p value < 0.01 were used to consider a marker significantly associated with a trait. .. Regions showing

segregation distortion for SRA, referred to here as 'hotspots', were identified when more than five significant marker-trait associations were found within 5 cM. For each hotspot, the parental line most represented in terms of allele frequency in the 'narrow' tail populations was considered as donor for narrow SRA alleles.

Marker-assisted selection in an independent $F_{4:5}$ population

As segregation for SRA was clearly observed in Pop1 – Ma/Dr, seeds from the F_1 generation were progressed to the $F_{4:5}$ generation in parallel to the backcross populations, to develop an independent population for MAS tests (Figure 9). All generations were grown in the speed breeding system described above, except the F_2 generation, which was sown in the field as four rows in six meter plots at the University of Queensland Gatton Research Station, UQ, Gatton, Queensland, Australia (27.54°S 152.34°E, 89 metres above sea level). Single spikes from 52 F_2 selected plants were harvested green and dried using an air-forced dehydrator at ambient temperature. Following generations were all produced in the speed breeding system via single seed descent, resulting in 52 $F_{4:5}$ lines.

Genomic DNA was extracted from the 52 $F_{4:5}$ lines following the same protocol as described above. MAS, based on hotspots identified in Pop1 – Ma/Dr, was applied to the $F_{4:5}$ lines *in silico*. Five lines having the greatest total number of alleles for narrow SRA found in the hotspots and five lines having the lowest total number of alleles for narrow SRA were selected. Phenotyping for SRA was conducted as described in Chapter 3, using a randomized complete block design with six seeds of each of the 52 lines. SRA was analysed using a mixed model, containing 'Line' and 'Rep' as random components, and BLUPs were obtained as described above. A Welch two sample t-test was used to compare SRA means attained by the narrow and wide groups of $F_{4:5}$.

Results

Genotypic variability for seminal root angle

Phenotyping the panel of candidate parent wheat lines revealed a wide range in SRA in both experiments. In the first candidate parent phenotyping experiment (CandP–1), SRA ranged from 70° ± 6° for the narrowest candidate Spitfire, to 101° ± 5° for the widest candidate Suntop (Figure 11). In the second experiment (CandP–2), SRA ranged from 73° ± 5° for 36:ZWW11 to 110° ± 5° for Suntop (Figure 11). Despite variation for SRA between the two

experiments, the rank order of genotypes was quite similar, with 36:ZWW11, Drysdale, Dharwar Dry, Spitfire, and ZWW10-50 consistently exhibiting narrow SRA, and Hartog, Mace, Suntop, and Wallup wide SRA.

The six selected parental lines displayed contrasting SRA phenotypes, ranging from the narrowest to the widest: Dharwar Dry (75° ± 6°), Drysdale (76° ± 5°), SB062 (81° ± 6°), Scout (92° ± 6°), Mace (94° ± 6°), and Suntop (101° ± 5°) in the first experiment, and Drysdale (77° ± 5°), Dharwar Dry (80° ± 6°), Scout (88° ± 5°), SB062 (95° ± 5°), Mace (100° ± 5°), and Suntop (110° ± 5°) in the second experiment (Figure 11).

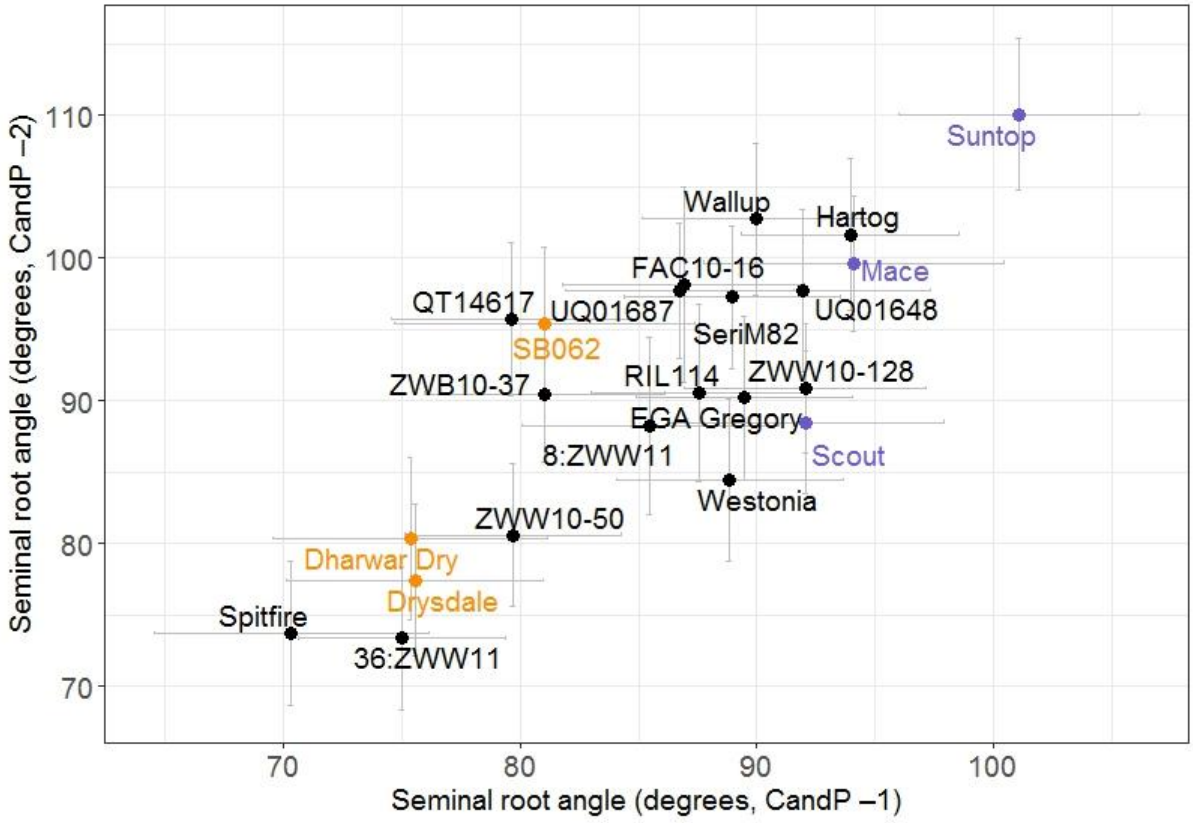

Figure 11: Mean seminal root angle (BLUPs) of the panel of 22 Australian-adapted wheat lines tested as candidate parents for phenotypic selection experiments

The panel was evaluated in two experiments, namely CandP–1 (n = 3 – 9) and CandP–2 (n = 3 – 9). The panel includes the donor lines (tan) and the recurrent parents (purple) selected to develop the backcross populations in this study. Error bars in grey represent the standard errors of the means.

Comparison of population distribution in tail populations

Following selection for SRA in the BC_1F_2 generation (with a 21-24% selection intensity, Table 5), assessment of the BC_1F_3 progeny representing 'narrow' and 'wide' tails within each backcross population revealed extensive overlap in SRA phenotypes and little, shift in

distribution (Figure 10). Further, no significant differences were found between the population SRA means (Table 6). Within each 'narrow' tail BC_1F_3 population, the lower and upper quartiles ranged from 65 to 91° for Pop1 – Ma/Dr; from 67 to 88° for Pop2 – Su/Dh, and from 58 to 78° for Pop3 – Sc/SB. Within each 'wide' tail population, the lower and upper quartiles ranged from 68 to 93° for Pop1 – Ma/Dr; from 67 to 89° for Pop2 – Su/Dh; and from 58 to 82° for Pop3 – Sc/SB. The SRA averaged 79° ± 17°, 68° ± 15° and 77° ± 14° in 'narrow' tail populations of Pop1 – Ma/Dr, Pop2 – Su/Dh, and Pop3 – Sc/SB, respectively (Table 6, represented by pink dash lines in Figure 10). There was little change in the 'wide' tail populations where the SRA averaged 80° ± 18°, 69° ± 17°, and 78° ± 15° for Pop1 – Ma/Dr, Pop2 – Su/Dh, and Pop3 – Sc/SB, respectively (Table 6, represented by blue dash lines in Figure 10).

Table 6: Comparison of mean seminal root angles between tail populations

Means are presented for tail populations selected for narrow or wide seminal root angle (SRA) from three backcross populations, Pop1 – Ma/Dr (Mace/Drysdale//Mace), Pop2 – Su/Dh (Suntop/Dharwar Dry//Suntop), and Pop3 – Sc/SB (Scout/SB062//Scout) in the BC_1F_3 and BC_1F_4 generations. The p-values from a Welch two sample t-test is displayed for each comparison between tail populations selected for narrow and wide SRA. Significance: ns, non-significant at P = 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Generation	Population	Mean root angle for tail populations		P-value
		Narrow	Wide	
BC_1F_3	Pop1 – Ma/Dr	79°	80°	0.4 (ns)
	Pop2 – Su/Dh	77°	78°	0.5 (ns)
	Pop3 – Sc/SB	68°	69°	0.4 (ns)
BC_1F_4	Pop1 – Ma/Dr	81°	91°	$1.6.10^{-7}$ (***)
	Pop2 – Su/Dh	80°	90°	$1.9.10^{-6}$ (***)
	Pop3 – Sc/SB	84°	82°	0.5 (ns)

Following a second cycle of selection in the BC_1F_3 generation (27-31% selection intensity, Table 5), assessment of the BC_1F_4 progeny representing 'narrow' and 'wide' tails within each backcross population revealed significant differences in SRA for Pop1 – Ma/Dr and Pop2 – Su/Dh, but not for Pop3 – Sc/SB (Table 6 and Figure 10). For Pop1 – Ma/Dr, the mean SRA was 81° ± 18° for the 'narrow' tail and 91° ± 17° for the 'wide' tail. This represented a significant change of 10° as a result of bi-directional selection performed in the BC_1F_2 and BC_1F_3 generations (Table 6). Similarly for Pop2 – Su/Dh, the mean SRA was 80° ± 18° for 'narrow' tail and 90° ± 17° for the 'wide' tail; also providing a significant difference of 10° (Table 6). For Pop3 – Sc/SB, the population with parents having intermediate SRA phenotypes, no significant difference was found between the SRA means of the two tail

populations (Table 6). The phenotypic distribution of raw SRA values revealed similar patterns. For instance, the lower and upper quartiles ranged from 70 to 93° and 80 to 104° for ‘narrow’ and ‘wide’ tails of Pop1 – Ma/Dr, respectively; from 69 to 92° and 80 to 101° for ‘narrow’ and ‘wide’ tails of Pop2 – Su/Dh, respectively; and from 68 to 99° and 70 to 96° for ’narrow’ and ‘wide’ tails of Pop3 – Sc/SB, respectively.

For Pop1 – Ma/Dr and Pop2 – Su/Dh, the difference between ‘narrow’ and ‘wide’ tail populations at the BC_1F_4 generation compared to the to the BC_1F_3 generation was due to wider mean SRA in the ‘wide’ tail populations (11° and 13° wider, respectively), while SRA remained almost constant in the ‘narrow’ tail populations (2° and 3° wider, respectively; Table 6). Interestingly for Pop3 – Sc/SB where little differentiation between narrow and wide tails was observed, mean SRA for the ‘narrow’ and ‘wide’ tails were 16° and 13° wider, respectively, when comparing BC_1F_4 to BC_1F_3 generation.

Phenotyping of the six parental lines also revealed some variation between experiments performed in each generation of phenotypic screening (Figure 10 and Figure 12). All parental lines were 7° wider on average when assessed as part of the phenotypic screening of the BC_1F_4 generation compared to the BC_1F_3 generation, and 3° narrower in average when assessed as part of the phenotypic screening of the BC_1F_3 generation compared to the BC_1F_2 generation (Figure 10 and Figure 12). However, the rank between parental lines was maintained across experiments, with Drysdale and Dharwar Dry exhibiting the narrowest SRA, Mace and Suntop the widest, and Scout and SB062 intermediate (Figure 10). This rank consistency was expected as a common seed source of each parental line was used as a benchmark, and not subjected to selection for SRA.

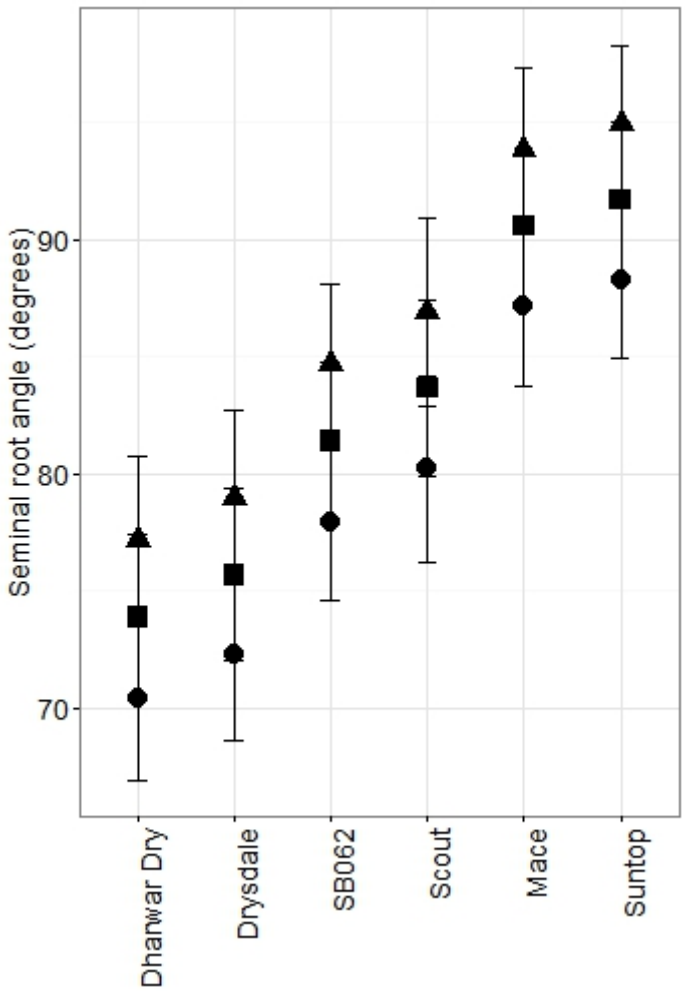

Figure 12: Seminal root angle of the six parental lines assessed in the three phases of phenotyping
Parental lines were included at each generation of phenotypic screening of the backcross lines: within the BC_1F_2 (square, n = 6 – 9), within the BC_1F_3 (circle, n = 4 – 9) and within the BC_1F_4 (triangle, n = 4 – 9). The error bars display standard errors.

Characterisation of fixed lines selected via direct phenotypic selection

Fixed backcross lines ($BC_1F_{4:5}$) with extreme narrow phenotypes for SRA from Pop1 – Ma/Dr only were compared to their associated parental lines Drysdale and Mace to validate the shift in SRA observed after three cycles of selection. SRA for the selected 46 $BC_1F_{4:5}$ lines from the narrow tail of Pop1 – Ma/Dr ranged from 62° ± 5° to 83° ± 4°, and averaged 74° ± 4° (data not shown). The ten narrowest lines ranged from 62° ± 5° to 70° ± 4° (Figure 13). As in previous experiments, SRA for parental lines were contrasting, with Drysdale the narrowest (72° ± 5°) and Mace the widest (85° ± 5°; Figure 13). Interestingly, the ten $BC_1F_{4:5}$ backcross lines were not significantly different from the donor parent Drysdale but were significantly narrower than Mace, the recurrent parent (Figure 13). The narrowest line (Pop1 – Ma/Dr –01) was 23^o narrower than Mace (i.e. a shift of - 27% in SRA).

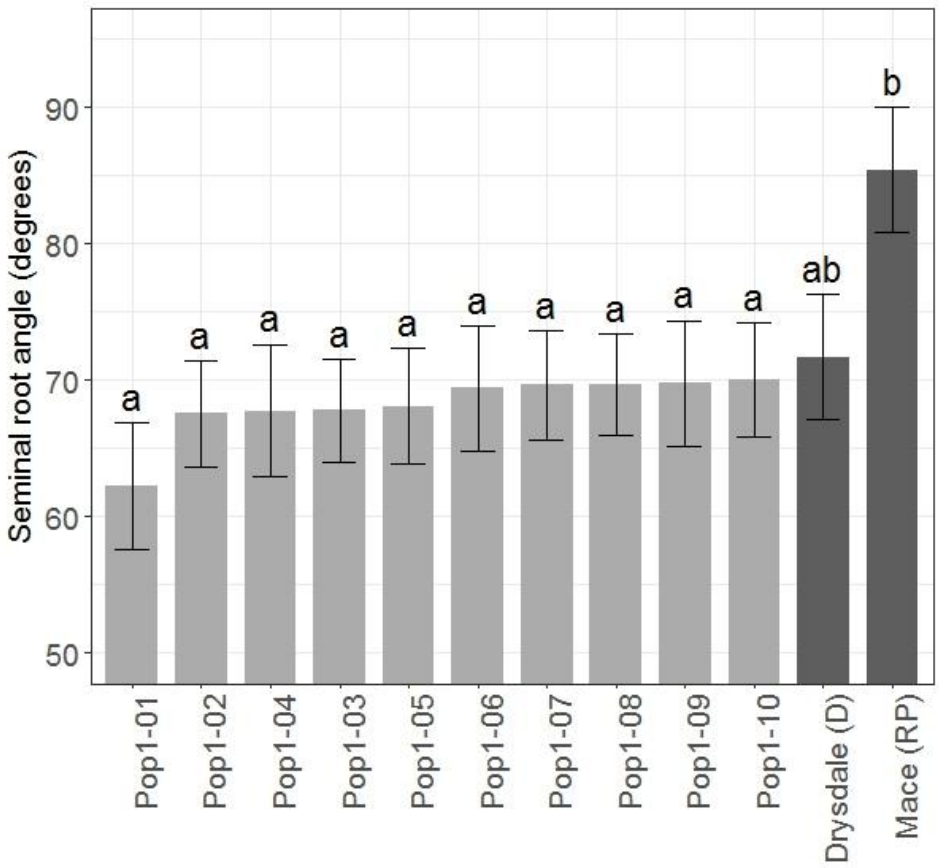

Figure 13: Seminal root angle for selected $BC_1F_{4:5}$ lines and respective parents

Seminal root angle (SRA) was measured for ten $BC_1F_{4:5}$ lines displaying the narrowest SRA from Pop1 – Ma/Dr (light grey) and associated parents (dark grey), i.e. the recurrent parent (RP) Mace and the donor line (D) Drysdale. Letters represent groups according to a Fisher's LSD test (p-value = 0.05). Error bars represent the standard errors of the means in this experiment (n = 5 – 10).

Comparison of allele frequency in tail populations

Allele frequency from the recurrent parent varied between 0 and 100% along the genome in both 'narrow' and 'wide' tail populations of Pop1 – Ma/Dr and Pop3 – Sc/SB (data not shown). Hotspots showing segregation distortion for SRA were identified and parental donors for narrow SRA identified for each. These hotspots included between 16 and 48 marker loci for Pop1 – Ma/Dr, and between 18 to 107 marker loci for Pop3 – Sc/SB (data not shown). In total, eight hotspots were identified in Pop1 – Ma/Dr (*hp1.Sra* - *hp8.Sra*) and five in Pop3 – Sc/SB (*hp9.Sra* - *hp13.Sra*, Table 7). Among these 13 hotspots, *hp2.Sra* discovered in Pop1 – Ma/Dr and *hp10.Sra* discovered in Pop3 – Sc/SB overlapped on chromosome 2B. The other 11 hotspots had locations on the genome that were unique to each population (Table 7).

Table 7: Hotspots identified through comparative frequency analysis

Comparison of marker frequency between the 'narrow' and 'wide' tail populations of Pop1 – Ma/Dr (Mace/Drysdale//Mace) and Pop3 – Sc/SB (Scout/SB062//Scout) at the BC_1F_4 generations revealed regions under selection for seminal root angle (SRA). Two hotspots occurring in Pop1 – Ma/Dr and Pop3 – Sc/SB at overlapping regions on chromosome 2B are highlighted in bold.

Population	Hotspot	Chromosome	Bottom position (cM)	Top position (cM)	Number of significant markers	Origin of the allele for narrow SRA
Pop1 – Ma/Dr	*hp1.Sra*	1A	6.2	13.9	24	Mace
	hp2.Sra	**2B**	**73.7**	**80.8**	**48**	**Drysdale**
	hp3.Sra	3A	12	19	20	Drysdale
	hp4.Sra	3B	13.5	32.5	23	Drysdale
	hp5.Sra	3D	137.6	151.1	40	Mace
	hp6.Sra	5A	59.5	78.2	25	Mace
	hp7.Sra	7A	74.4	97.6	33	Drysdale
	hp8.Sra	7D	78.5	97.3	16	Mace
Pop3 – Sc/SB	*hp9.Sra*	1D	25.3	57.5	30	Scout
	hp10.Sra	**2B**	**62.5**	**82.6**	**44**	**Scout**
	hp11.Sra	4A	19.8	30.9	107	SB062
	hp12.Sra	6A	97.6	100.8	37	Scout
	hp13.Sra	6B	2.4	9.3	18	Scout

In Pop1 – Ma/Dr, 50% of the narrow SRA alleles originated from the recurrent parent Mace while 80% came from Scout in Pop2 – Su/Dh (Table 7). Backcross lines displayed different combinations of alleles for narrow and wide SRA alleles at each marker loci. For example in Pop1 – Ma/Dr, some backcross lines from the 'narrow' tail population displayed some alleles for wide SRA, while some backcross lines from the 'wide' tail population displayed some alleles for narrow SRA (Figure 14).

Figure 14: Heatmap of the alleles for narrow and wide seminal root angle

Representation of the alleles for narrow and wide seminal root angle (SRA) at the eight hotspots, comprising between 16 and 48 marker loci, detected in the 49 lines from the 'wide' and the 46 lines from the 'narrow' tail populations of Pop1 – Ma/Dr (Mace/Drysdale//Mace). The parent contributing the allele for narrow seminal root angle at each hotspot is indicated in brackets. White indicates that the source for an allele in a particular line is unassigned.

Characterisation of fixed lines selected via marker-assisted selection

Characterisation for SRA in the independent $F_{4:5}$ population derived from the same parental lines as Pop1 – Ma/Dr (i.e. Mace and Drysdale) revealed phenotypic variation ranging from 67° ± 5° to 90° ± 5° (Figure 15). Of the group of five lines selected as having the greatest total number of alleles for narrow SRA (i.e. five or six alleles out of eight), SRA ranged from 71° ± 5° to 81° ± 5°, and averaged 76° ± 5° (Figure 15). Of the group of five lines selected as having the lowest total number of alleles for narrow SRA (i.e. zero or one allele out of eight), SRA ranged from 76° ± 5° to 90° ± 5°, and averaged 84° ± 5° (Figure 15). A Welch

two sample t-test between the two groups indicated a significant difference of 8° (p-value = 0.04). However in this population, the narrowest line (67° ± 5°) which had four alleles for narrow SRA, and the widest line (90° ± 5°) which had three alleles for narrow SRA, were found among the unselected lines (Figure 15).

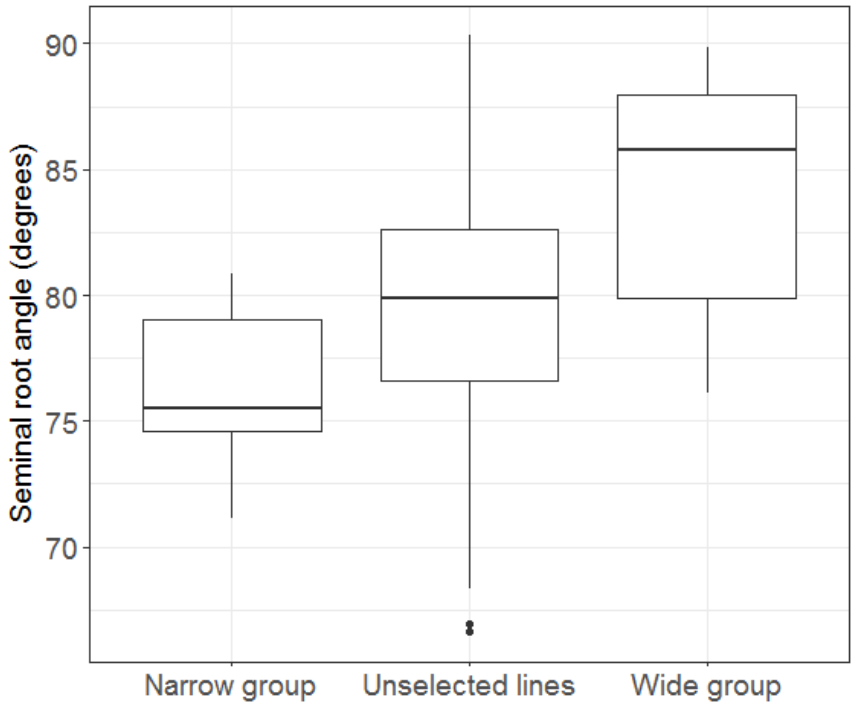

Figure 15: Phenotypic variation for seminal root angle of an independent population

Box and whisker plots of seminal root angle of 52 $F_{4:5}$ lines from a population derived for Mace x Drysdale independently of the selected tail populations described above. Five lines presenting the greatest total number of alleles for narrow SRA are represented in the 'narrow group' (left), while the five presenting the lowest total number of alleles for narrow SRA are represented in the 'wide group' (right). The remaining 42 $F_{4:5}$ lines are represented in the 'unselected lines' (centre). The bottom and the top of the boxes display the first and third quartile values. The band inside the box displays the median and the ends of the whiskers display the minimum and maximum values.

Discussion

We believe this to be the first report of direct phenotypic and molecular selection for root system architecture in early generations of a crop species. We applied bi-directional selection in the BC_1F_2 and BC_1F_3 generations, which successfully shifted the mean SRA by 10° in two wheat populations segregating for the trait. By combining efficient phenotyping and rapid generation advance, backcross-derived lines ($BC_1F_{4:5}$) enriched with alleles for narrow SRA were developed within 18 months. Further, application of MAS in an independent population successfully identified five lines with narrow SRA. We propose that

a similar root trait-based approach could be implemented in breeding programs to assist the development of high yielding cultivars with adapted root architecture.

Useful genotypic diversity for seminal root angle was identified

The panel of 22 candidate parental wheat lines evaluated in this study revealed a high degree of phenotypic variation for SRA, suggesting there are valuable sources of genetic diversity that can be exploited to improve root system architecture in breeding programs. In this panel, wheat genotypes displayed variation of 34° for SRA, with mean phenotypes for two experiments ranging from 72 to 106°. In Chapter 3, a panel of 24 spring wheat lines was also characterised for SRA using the clear-pot method. However, narrower phenotypes and a smaller range was observed (60 to 84°, i.e. a range of 24°). Manschadi et al. (2008) used the gel-filled chamber method to characterise a collection of 30 wheat genotypes for SRA, including some in common with the panel assessed in Chapter 3, and reported a range from 72 to 112° (i.e. a range of 40°). Notably, of the lines that were common in studies by Manschadi et al. (2008) and in Chapter 3, genotypes displaying extreme phenotypes (i.e. narrowest and widest) were largely in agreement despite differences in screening methods. It seems likely that the range in SRA reported in these studies may not represent the full extent of genetic variation in wheat germplasm, as the panels mostly comprised spring wheats from CIMMYT and Australia, some of which share similar genetic backgrounds. In comparison, barley (*Hordeum vulgare* L.) appears to display a broader range in SRA phenotypes: Robinson et al. (2016) reported a range from 13 to 82° (i.e. a range of 69°) using the clear-pot method for a panel of 30 Australian cultivars and breeding lines.

Segregating populations adapted to Australian environments were developed

The three populations examined in this study were developed for their relevance to Australian wheat breeders. The three recurrent parents Mace, Scout, and Suntop, are high-performing cultivars widely grown throughout the western, southern, and eastern production regions of the Australian wheat-belt, respectively. The three donor lines Drysdale, SB062 and Dharwar Dry, combine drought and heat adaptation traits, which are considered desirable for improving and expanding wheat production in Australia.

The six parental lines displayed contrasting SRA phenotypes, with narrow to intermediate root angle for the three donor lines, and intermediate to wide angle for the three recurrent lines. Crosses for Pop1 – Ma/Dr and Pop2 – Su/Dh were selected with the intention of introgressing beneficial alleles for narrow SRA from donors with narrow SRA (Drysdale and

Dharwar Dry) to locally-adapted cultivars with wide SRA (Mace and Suntop). For Pop3 – Sc/SB, the cross was selected to test for transgressive segregation for SRA using donors contrasting genetically, but which have similar intermediate root angles.

Seminal root angle was modified by selection

We examined the phenotypic distribution of SRA over the course of selection for either narrow or wide SRA in segregating generations. After one cycle of selection, there was no significant difference between the distributions of SRA for the 'narrow' and 'wide' tails within each of the three backcross populations. However, following two cycles of selection (i.e. BC_1F_2 and BC_1F_3 screens), a significant shift of approximately 10° was observed between SRA distributions of the 'narrow' and 'wide' tails for two of the three backcross populations (i.e. Pop1 – Ma/Dr and Pop2 – Su/Dh). While contrasting SRA phenotypes were displayed by parents for Pop1 – Ma/Dr and Pop2 – Su/Dh, the donor parent and recurrent parent for Pop3 – Sc/SB both displayed intermediate SRA. Thus, in this study, phenotypic selection in early generations for SRA was only effective when applied to populations derived from parents that were phenotypically distinct.

Significant shifts observed in the two backcross populations were due to the 'wide' tail population getting wider. These results could suggest that it is easier to select for wide SRA than further reducing SRA to produce narrower phenotypes. However, wider phenotypes were observed in all experiments performed at the BC_1F_4 generation of phenotypic screening compared to previous generation. For example, both 'narrow' and 'wide' tail populations of Pop3 – Sc/SB, and phenotypes displayed by parental lines, were wider when assessed at the BC_1F_4 generation compared to the BC_1F_3 generation of phenotypic screening. While effort was made to minimise variation in environmental factors across experiments, some variation in results across experiments could be attributable to subtle differences in temperature, water and or nutrient content; all of which are known to influence root growth (Al-Khafaf *et al.*, 1989; Vincent and Gregory, 1989; Adalsteinsson, 1994). Hence, results should be compared between experiments in relative terms rather than in absolute values. While a shift was clearly observed in the latter generation of population development, we cannot on the basis of the current evidence determine whether this shift was attributed to 'wide' tail populations getting wider, 'narrow' tail populations getting narrower, or both.

We compared allele frequency between tail populations selected for narrow and wide SRA in two of the three backcross populations: firstly, in Pop1 – Ma/Dr where a significant shift in phenotype was observed for the selected tail populations and secondly for Pop3 – Sc/SB, where this did not occur. In both populations, phenotypic selection had increased the allele frequency for narrow SRA in the 'narrow' tail populations, and decreased the allele frequency for narrow SRA in the 'wide' tail populations. Where a significant shift for SRA was observed in Pop1 – Ma/Dr, eight regions under selection for SRA were identified. Interestingly in Pop3 – Sc/SB, where no shift was observed for SRA, five regions under selection for SRA were still identified, including one in common with Pop1 – Ma/Dr.

Previous studies in wheat suggest that genetic variation for SRA could be governed by multiple genes, each with minor effect (Liu *et al.*, 2013; Christopher *et al.*, 2013). Thus, parental lines may have contributed different alleles for SRA at a number of loci. Regions under selection identified in Pop3 – Sc/SB may have smaller effects compared to those identified in Pop1 – Ma/Dr. This could explained why despite a clear shift in allele frequencies in Pop3 – Sc/SB, no phenotypic shift was observed in tail populations for narrow and wide SRA. However, further studies are required to estimate allele effects and confirm this hypothesis.

The source of alleles contributing narrow SRA in the 'narrow' tail populations of Pop1 – Ma/Dr and Pop3 – Sc/SB were both donor and recurrent parental lines. Interestingly, Mace which displayed wide SRA, contributed half of the alleles for narrow SRA in Pop1 – Ma/Dr. This tends to confirm that SRA is under complex genetic control, possibly involving epistatic, additive, antagonist, and/or synergetic genetic effects. If this is the case, it could help to explain why particular combinations of alleles for narrow SRA may result in different phenotypes.

Opportunities for breeding for seminal root angle

The 46 $BC_1F_{4:5}$ backcross lines derived from the 'narrow' tail of Pop1 – Ma/Dr displayed a SRA average (76°) closer to the donor Drysdale (72°) than to the recurrent parent Mace (85°). Among those 46 selected $BC_1F_{4:5}$, ten lines had a SRA significantly narrower than their respective recurrent parent (Mace). These results demonstrate how repeated cycles of selection (three here) for narrow SRA in early generations can shift trait values. The ten $BC_1F_{4:5}$ lines developed in this study combined favourable alleles for narrow SRA in this elite

background and could thus be directly used in breeding programs for top-crossing or for further testing in the field.

The five lines from the independent set of unselected $F_{4:5}$ lines that were subsequently selected for narrow SRA through MAS displayed a mean root angle significantly narrower than the five $F_{4:5}$ lines selected for wide SRA from this set (difference of 8°). This result indicates potential for molecular selection in early generations of wheat to combine favourable alleles for SRA in breeding lines. Yet, there were also lines in this set which displayed extreme phenotype for narrow and wide SRA but were not selected via MAS. Some regions influencing SRA might not have been identified through comparative marker frequency analysis due to the small population size. Alternatively, the dominant marker system may have caused some regions which were still segregating to be indicated as fixed, influencing the marker analysis. Regions influencing SRA used for MAS were likely to be specific to this population. Hence, further genetic studies using large multi-parent populations that incorporate high genetic diversity and recombination events are needed to detect marker-trait association to fully exploit the potential of MAS.

In wheat, it has previously been shown that changes in root system architecture affect the spatial and temporal pattern of water extraction and can potentially lead to yield increases in summer dominant rainfall regions (Manschadi *et al.*, 2006; Wasson *et al.*, 2012), and possibly also regions with Mediterranean climates (McDonald, 2010). For example, Manschadi et al. (2010) found that the narrower root system in the drought tolerant CIMMYT line SeriM82 corresponding with a narrower SRA (i.e. 72°) also corresponded with greater root length density at depth and higher yield, compared to the Australian cultivar Hartog which had a wider SRA (i.e. 104°). Therefore, the 46 $BC_1F_{4:5}$ backcross lines with SRA ranging from 49 to 87° and the five $F_{4:5}$ lines with SRA ranging from 71 to 81° may also lead to narrower root systems and higher yields in some environments. However, further research is required to quantify the impact of SRA on the temporal and spatial patterns of water uptake and grain yield in these lines. Field studies are needed to determine the value of narrow and wide root system architectural traits in targeted environments which typically vary in soil type and rainfall patterns (Potgieter *et al.*, 2002; Chenu *et al.*, 2013), especially given the strong influence of soil structure on root growth and distribution at depth (White and Kirkegaard, 2010).

Conclusion

Breeding directly for favourable root system architecture in wheat has been limited by the availability of suitable phenotyping methods. In this chapter, we used the method described in Chapter 3 to rapidly develop lines enriched with alleles for narrow SRA. The clear-pot method developed in Chapter 3, which was designed to provide heritable, precise and reproducible phenotypic information on the seedling roots, was used here for rapid SRA screening at early growth stages, out-of-season, and in a more homogeneous environment than the field. The benefit of performing selection in early generations is that, once a beneficial root ideotype has been identified, individuals with undesirable gene combinations can be eliminated early in the breeding cycle, thereby allowing breeders to advance a smaller set of plants enriched with the target trait. Thus, expensive field-testing is targeted to a potentially superior set of inbred lines. As only two cycles of selection were required to shift population distribution for SRA, this strategy allows breeders to rapidly enrich their germplasm with favourable alleles for root system architecture. This could be readily integrated into breeding programs aimed at improving wheat yield in environments that often experience terminal drought stress, for example. We also investigated regions influencing seminal root traits and prospects for MAS. Though promising, this study was restricted to bi-parental populations. Genetic studies using multi-parent populations will be required to tackle such complex traits and to identify marker-trait associations useful for application in breeding programs.

Acknowledgment

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Chapter 5

A multi-reference parent nested association mapping population for dissecting the genetic controls of seminal root traits in wheat

Abstract

In Chapter 4, we showed allele frequency for seminal root angle could be shifted in early generations of wheat (*Triticum aestivum* L.). We identified genetic regions influencing this proxy trait, though the study was limited to bi-parental populations. We suggested multi-parent populations would provide higher power and greater resolution for mapping quantitative trait loci (QTL) for complex root traits. In this chapter, we investigated the genetic architecture of seminal root angle and number, using a multi-reference parent nested-association mapping (MR-NAM) population. To identify relevant QTL for breeders and facilitate their introgression into elite germplasm, the MR-NAM population was developed by nesting 11 diverse founders within three cultivars preferred for the western, southern, and eastern production regions of the Australian wheat belt. Founders were selected based on target traits involved in drought and heat adaptation, disease resistance and acid soil-tolerance. Several founders had root architectural traits associated with adaptation to these stresses. An incomplete factorial design crossing scheme was used to generate 15 families comprising a total of 612 $F_{4:5}$ NAM lines. Using the 'clear pot' phenotyping method described in Chapter 3, the 15 families were evaluated for seminal root angle and number. Both traits displayed high heritability and a high degree of variation, within and across families. Genome wide association mapping identified 29 QTL for seminal root traits, each with small to moderate effect. Among these newly identified QTL, nine were overlapping with genomic regions previously identified in wheat. This improved understanding of the genetic control of root traits in wheat will aid breeders to combine desirable traits in elite germplasm adapted to water-limited environments.

Introduction

Although numerous marker-trait associations have been reported in the literature for many crop species, few examples of successful exploitation of these mapped quantitative trait loci (QTL) in breeding programs have been reported (Xu and Crouch, 2008; Bernardo, 2008).

This is because QTL information can be difficult to extrapolate from specific mapping populations to highly selected breeding populations. The genetic base of breeding populations is often narrow, allele frequencies are affected by segregation distortion, and many genomic regions are fixed due to selection (Würschum, 2012). As a consequence, QTL mapping performed in bi-parental populations based on crosses between elite and exotic material might lead to the identification of QTL that are absent or do not segregate in breeding populations. Further, QTL effects detected in bi-parental populations may be reliant on other gene(s) in that specific genetic background. Hence, QTL identified using exotic donors may also lack function when deployed in elite genetic backgrounds. Similarly, association mapping applied to diversity panels might lead to the discovery of QTL within the context of relatively unadapted germplasm that may not be relevant for breeding purposes (Breseghello and Sorrells, 2006). Hence, the use of suitable mapping populations to provide useful information for both geneticists and plant breeders is crucial for successful marker-assisted selection (MAS).

Multi-parent populations such as nested association mapping (NAM) and multi-parent advanced generation inter-cross (MAGIC) have been used to detect QTL for complex polygenic traits in elite backgrounds (Huang *et al.*, 2012; Bandillo *et al.*, 2013; Maurer *et al.*, 2015). By exploiting multiple diverse parents, these populations offer an opportunity to evaluate multiple alleles at any locus at the same time, and investigate interactions between loci. In the NAM approach, a panel of founders is crossed or backcrossed to a single common parent to produce a large number of recombinant inbred lines (RILs) from each cross. The common parent of a NAM population enables the study of QTL by genetic background interaction (Blanc *et al.*, 2006). The NAM approach also provides an appropriate context to evaluate the merit of diverse alleles, in an adapted genetic framework. Therefore, NAM populations are ideal for studying genetics in diverse sets of environments and also breeding for adaptation to them. In this way they can help to connect gene discovery to gene introgression in elite germplasm.

In this chapter, we proposed extending the NAM approach to construct a multi-reference parent nested association mapping (MR-NAM) population to investigate the genetic architecture of seminal root angle (SRA) and seminal root number (SRN) in wheat (*Triticum aestivum* L.). A MR-NAM population is obtained by crossing a panel of diverse founders to multiple reference parents as opposed to a single reference parent for a conventional NAM population. The MR-NAM population is therefore comprised of multiple NAM populations

that have some founders in common. We provided what we believe to be the first report of the development, characterisation, and utilisation of a MR-NAM wheat population, and demonstrated its power for genetic analysis when applied to wheat seminal root traits.

Materials and methods

Plant material

Three Australian spring wheat cultivars were used as reference parents of the MR-NAM population. Notably, these three cultivars, Mace, Scout, and Suntop, were also the recurrent parents used in Chapter 4 to develop the three backcross populations. All three cultivars have high wheat quality classifications and a high level of disease resistance, including to leaf rust and stem rust (www.dpi.nsw.gov.au).

Eleven founder lines were selected on the basis of the combination of attributes which are desirable for improving and expanding wheat production in diverse environments such as those found in the Australian wheat belt (Table 8). Among the founders, Dharwar Dry, Drysdale, and SB062, were also donor parents of the three backcross populations detailed in Chapter 4. Dharwar Dry and SeriM82 are both known to have a dense root system at depth (Manske and Vlek, 2002; Manschadi *et al.*, 2006; Christopher *et al.*, 2008) and stay-green phenotype (Olivares-Villegas *et al.*, 2007; Christopher *et al.*, 2008; Manschadi *et al.*, 2010). Drysdale is an Australian cultivar with superior transpiration efficiency (Condon *et al.*, 2004; Tausz-Posch *et al.*, 2012). SB062 is a breeding line developed in the 'Physiological Breeding' program led by Dr Matthew Reynolds at the International Maize and Wheat Improvement Center (CIMMYT), with tolerance to warm conditions (Chenu et al. unpublished), low canopy temperature, and high levels of water soluble carbohydrates (Olivares-Villegas *et al.*, 2007; Dreccer *et al.*, 2009). The three CIMMYT lines, ZWB10-37, ZWW10-128, and ZWW10-50, were selected for high yield in the CIMMYT-Australia-ICARDA Germplasm Evaluation (CAIGE) trials conducted in Australia. Cultivars EGA Gregory and EGA Wylie were selected from the breeding program at the Queensland Department of Agriculture and Fisheries (QDAF) for multiple disease resistance. EGA Gregory has high resistance to root lesion nematodes (*Pratylenchus thornei*), while EGA Wylie has high levels of *Fusarium* crown rot resistance and black point tolerance (Queensland Wheat Variety Guide, 2014; Zheng *et al.*, 2014). The Australian cultivar Westonia was selected for acid soil tolerance, and tolerance to manganese and aluminium

toxicities in soil (Tang *et al.*, 2003; Khabaz-Saberi *et al.*, 2010). Finally, the Australian line RIL114 was selected for pre-harvest sprouting tolerance, with high levels of grain dormancy (Hickey *et al.*, 2009).

Table 8: Main criteria of choice and main related agronomic traits of the 11 wheat founders used in this study to create the multi-reference parent nested association mapping population

Name	Reason for selection	Target traits of interest
Dharwar Dry	Drought adaptation features	Adapted to rainfed wheat production in India, deep root system, stay-green phenotype (Manske and Vlek, 2002; Manschadi *et al.*, 2008)
Drysdale	Heat tolerance	High transpiration efficiency (Condon *et al.*, 2004; Tausz-Posch *et al.*, 2012)
EGA Gregory	Adaptation to nematodes	High resistance to root lesion nematodes (*Pratylenchus thornei*, Queensland Wheat Variety Guide, 2014)
EGA Wylie	Disease resistance	High levels of *Fusarium* crown rot resistance and black point tolerance (Queensland Wheat Variety Guide, 2014, Zheng *et al.*, 2014)
RIL114	Pre-harvest sprouting tolerance	High levels of grain dormancy donated by DH70 parent (Hickey *et al.*, 2009)
SB062	Heat tolerance	High levels of water soluble carbohydrates (Olivares-Villegas *et al.*, 2007; Dreccer *et al.*, 2009)
SeriM82	Drought adaptation features	Deep root system (Manschadi *et al.*, 2006, 2008), stay-green phenotype (Olivares-Villegas *et al.*, 2007; Christopher *et al.*, 2008; Manschadi *et al.*, 2010)
Westonia	Adaptation to sub-soil constraints	High tolerance to manganese and aluminium toxicities (Tang *et al.*, 2003; Khabaz-Saberi *et al.*, 2010) and wide international adaptation (Mathews *et al.*, 2006)
ZWB10-37	General adaptation	High yielding in CAIGE[2] trials conducted in South Australia
ZWW10-50	General adaptation	High yielding in CAIGE[2] trials conducted in Western Australia
ZWW10-128	General adaptation	High yielding in CAIGE[2] trials conducted in South Australia

[2]CIMMYT-Australia-ICARDA Germplasm Evaluation (CAIGE)

Selection of the 11 founders also considered pedigree and origin information in order to maximise genetic diversity (Table 9). Out of the 11 founders, six cultivars and elite breeding lines were developed at CIMMYT and five cultivars and elite breeding lines were developed by different Australian breeding programs (Table 9).

Table 9: Origin and pedigree of the 11 founders and three references used to develop the multi-reference parent nested association mapping population

Name	Type	Breeder[1]	Pedigree
Dharwar Dry	Cultivar	India	DWR39/C306//HD2189
Drysdale	Cultivar	CSIRO	Hartog*3/Quarrion
EGA Gregory	Cultivar	EGA	Pelsart/2*Batavia
EGA Wylie	Cultivar	EGA	QT 2327-1/Cook//Jupateco F 73/TR 590
Mace	Cultivar	AGT	Wyalkatchem/Stylet//Wyalkatchem
RIL114	Elite breeding line	UQ	UQ01484/RSY10//H45
SB062	Elite breeding line	CIMMYT	Seri M82/Babax
Scout	Cultivar	LPB	Sunstate/QH71-6//Yitpi
SeriM82	Elite breeding line	CIMMYT	Kavkaz/4/Saric F 70///Lerma Rojo 64A/Inia F66//Inia F66/Yecora F70/5/II-26992
Suntop	Cultivar	AGT	Sunco/2*Pastor//SUN436E
Westonia	Cultivar	Intergrain	Spica/Timgalen//Tosca/5/Wren:Mex//Ciano F 67/Noroeste F 66///Zambezi/4/Jacup*2/Bobwhite
ZWB10-37	Elite breeding line	CIMMYT	Tacupeto F2001/Brambling//Kiritati
ZWW10-128	Elite breeding line	CIMMYT	ESDA/KKTS
ZWW10-50	Elite breeding line	CIMMYT	Onix/4/Milan/Kauz//Prinia/3/BAV92

[1] Breeding program abbreviations: Australian Grain Technologies (AGT), International Maize and Wheat Improvement Center (CIMMYT), Commonwealth Scientific and Industrial Research Organisation (CSIRO), Enterprise Grains Australia (EGA), LongReach Plant Breeders (LPB), The University of Queensland (UQ)

All parental lines except the QDAF cultivar EGA Wylie were included in the panel of candidate parental lines assembled and characterised for SRA in Chapter 4 in two repeated experiments (CandP-1 and CandP-2). Parental lines of the MR-NAM population displayed variation of 30° for SRA. The two narrowest lines were the founders Drysdale (76°) and Dharwar Dry (78°) while the two widest lines were the reference parents Mace (97°) and Suntop (106°).

Pure seed for all cultivars and elite breeding lines was supplied by the Australian Grains Genebank based in Horsham, Victoria, Australia, with the exception of the elite breeding

lines RIL114 (supplied by Dr Hickey at The University of Queensland, Brisbane, Australia) and SB062 (supplied by Dr Scott Chapman at CSIRO, Brisbane, Australia).

Population development

The 11 founder lines were crossed to each of the three reference parents (Suntop, Scout and Mace) using an incomplete crossing scheme, producing a total of 15 F_1 crosses (Figure 16). The MR-NAM population consisted of three NAM populations: 'Ma-NAM' comprising four Mace-derived families, 'Sc-NAM' comprising five Scout-derived families, and 'Su-NAM' comprising six Suntop-derived families (Figure 16).

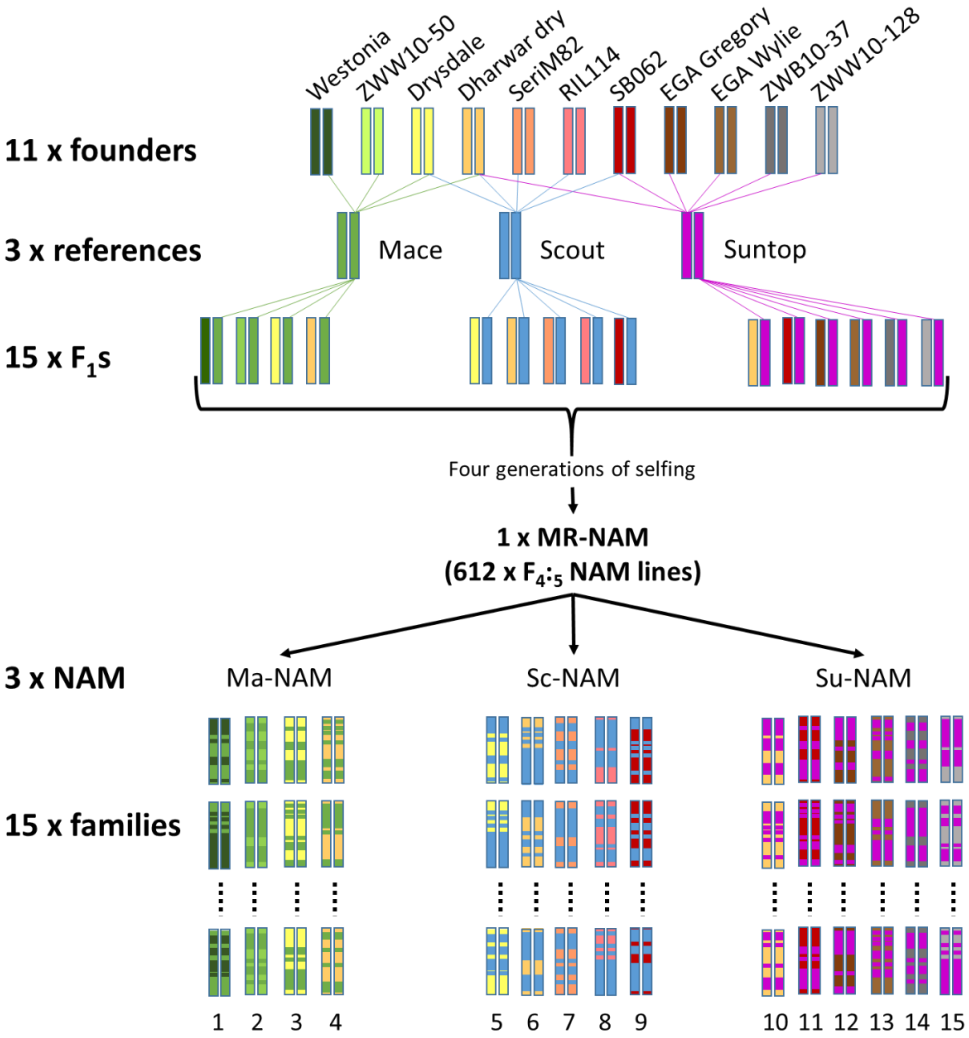

Figure 16: Diagram illustrating development of the multi-reference parent nested association mapping population.

Genome reshuffling occurred between 11 founders and three reference parents throughout crossing and development of recombinant inbred lines (RIL) via four generations of self-fertilizing. The final multi-reference parent nested association mapping (MR-NAM) comprised 15 families: four Mace-derived families (Ma-NAM), five Scout-derived families (Sc-NAM), and six Suntop-derived families (Su-NAM).

The final MR-NAM population comprised 612 NAM lines, from 15 families of 34 to 50 individuals (Table 10). The three NAM populations comprised 158 NAM lines in Ma-NAM, 213 NAM lines in Sc-NAM, and 241 NAM lines in Su-NAM (Table 10).

Table 10: Numbers of lines in families of the multi-reference parent nested association mapping classified according to the 15 families derived from three reference parents each adapted to one of three major Australian cropping regions and 11 founders donating specific traits of interest or adaptation to particular environments. The 15 families of multi-reference parent nested association mapping (MR-NAM) population are broken down according to common reference parents Mace (Ma-NAM), Scout (Sc-NAM), and Suntop (Su-NAM).

	Reference parents			
Founders	Mace	Scout	Suntop	***Total per founder***
Dharwar Dry	39	43	47	**129**
Drysdale	50	39	-	**89**
EGA Gregory	-	-	40	**40**
EGA Wylie	-	-	37	**37**
RIL114	-	42	-	**42**
SB062	-	49	44	**93**
SeriM82	-	40	-	**40**
Westonia	35	-	-	**35**
ZWB10-37	-	-	37	**37**
ZWW10-128	-	-	36	**36**
ZWW10-50	34	-	-	**34**
Total	**158 (Ma-NAM)**	**213 (Sc-NAM)**	**241 (Su-NAM)**	**612 (MR-NAM)**

During crossing, reference parents were used as females and founders were used as males. Crossing was performed using speed breeding as described in Chapter 4. Plants used for crossing were sown during January 2013. F_1 plants were rapidly grown in the speed breeding system to produce F_2 seed that was bulked at harvest.

In June 2013, approximately 200 seeds per F_2 population were then sown in the field, with each line sown into four rows in six meter plots at the University of Queensland Gatton Research Station, UQ, Gatton, Queensland, Australia (27.54^{o}S 152.34^o E, 89 metres above sea level). Replicated plots of each reference variety were also included. Wide plant spacing (36 cm row spacing) enabled field-based phenotypic selection for the key agronomic traits, plant height and maturity. To improve the agronomic quality of derived NAM lines, a moderate selection pressure for an 'appropriate agronomic window' was also applied, based on similarity to the respective reference parent. For instance, if an F_2 family was derived from a Mace by founder cross, selection was applied for plant height and maturity similar to that displayed by rows of Mace sown nearby within the trial. A maximum of 50 F_2 plants

were selected per family. In October 2013, single spikes from selected plants were harvested green and dried using an air-forced dehydrator at ambient temperature.

Populations were then subject to two generations of self-fertilization with single seed descent in each generation in the speed breeding system. Four $F_{3:}F_2$ lines were sown per 1.8L ANOVAPot® pot and grown under accelerated plant growth conditions with constant light and temperature, where water and nutrients were non-limiting. For the final generation, three F_4 plants per $F_{4:3}$ family were sown in large 4L ANOVAPot® pots. The larger pot volume provided plants with additional resources for tillering. To further enhance tiller development and maximise seed production, a diurnal (12 hour) photoperiod was adopted for the first four weeks of plant growth, before switching to constant light. One F_4 plant per pot was tagged for genotyping. Self-fertilized seed from this tagged plant was bulked to be phenotyped. By combining speed breeding technology and one generation of phenotypic selection, a total of 612 $F_{4:5}$ were successfully generated within only 18 months.

Molecular markers

DNA extraction and molecular marker genotyping were conducted as described in Chapter 4. A single F_4 plant representing each NAM line was sampled for leaf tissue, and DNA was isolated using the CTAB-based extraction protocol. Samples were genotyped with DArTseq markers using the wheat genome-by-sequencing platform, which returned scores for dominant SilicoDArTs markers (absence versus presence). Markers were positioned on the wheat DArT consensus map provided by Dr Andrzej Killian from DArT (http://www.diversityarrays.com/). The number of polymorphic markers with alleles occurring in more than 10% of genotypes within each NAM family ranged from 6,229 for Scout/SB062 to 8,998 for Suntop/Dharwar Dry (Appendix 2). This corresponded to a total number of 19,165 polymorphic markers for the MR-NAM population (specific to a family or in common across multiple families). Filtered markers were spanning the 21 chromosomes with an average of 2.6 markers per cM (Table 11).

Table 11: Total number of filtered SilicoDArT markers, number of unique positions, and marker coverage (unique position per cM) of the multi-reference parent nested association mapping population.

Chromosome	Chromosome length (cM)	Total number of markers	Unique position	Coverage per cM
1A	253	603	346	1.4
1B	284	1837	698	2.5
1D	137	415	180	1.3
2A	137	1273	407	3
2B	109	2315	781	7.1
2D	161	1012	269	1.7
3A	154	827	433	2.8
3B	160	1405	677	4.2
3D	151	467	200	1.3
4A	131	1151	399	3
4B	86	423	214	2.5
4D	96	109	60	0.6
5A	156	572	319	2
5B	154	1342	633	4.1
5D	154	236	125	0.8
6A	105	814	326	3.1
6B	93	1256	486	5.2
6D	113	299	153	1.4
7A	160	1110	459	2.9
7B	142	1290	464	3.3
7D	189	409	249	1.3

The 14 parental lines of the MR-NAM population, including the three reference parents and 11 founders, were also sent to DArT for genotyping. SilicoDArT markers were filtered to remove alleles occurring in less than 5% of genotypes, resulting in a subset of 23,796 positioned and un-positioned polymorphic markers.

Analysis of population diversity and structure

To investigate the relatedness of parental genotypes based on pedigree information, the coefficient of parentage (COP) was calculated based on the method of Kempthorne (1969). It was assumed that each parent contributes equally and any ancestors without known pedigrees are unrelated. COP were calculated using the International Crop Information System (Portugal et al., 2007).

To investigate the relatedness of parental genotypes based on marker information, a principal component analysis (PCA) was performed based on filtered SilicoDArT markers using TASSEL 5.2.33 (Bradbury *et al.*, 2007) and R software Version 3.3.1 (R Core team

2013). Similarly, the MR-NAM population structure was investigated by generating PCA based on filtered SilicoDArT markers, for the entire population and for each of the three NAM populations.

Pairwise linkage disequilibrium (LD) explained r^2 was determined for each of the three NAM populations using TASSEL 5.2.33. LD decay was measured as the genetic distance (cM) at which the squared correlation coefficient r^2 decayed to 0.2 and 0.3 on a logarithmic regression using R software Version 3.3.1.

Phenotyping for seminal root traits

Both SRA and SRN were assessed using the clear pot method described in Chapter 3. The experiment used a randomised complete block design where eight plants of each of the 612 NAM lines (and 47 lines including parents and checks) were randomised across 220 pots. Pots were placed on five benches, each bench containing 11 rows by four columns of pots. Constant temperature (17°C) was adopted over 24 hours with diurnal (12 h) natural light until SRA measurement at five days after sowing. Conditions were then changed to 22°C during the day (12 h) and 17°C during the night (12 h) until SRN measurement at 22 days after sowing. SRN was measured by pulling out the seedlings and counting the number of roots, as described in Chapter 3.

A linear mixed model, containing 'NAM line' as a fixed effect, was used to provide the best linear unbiased estimates (BLUEs). The mixed model contained random components that identified the structure of the experimental design for each experiment: 'Pot', and 'Column'. The variable 'Rep' and 'Row' had no significant effects and were removed from the analysis. Data were analysed with ASReml-R (Butler *et al.*, 2009) using R software Version 3.2.0 (R Core team 2013). A Welch two sample t-test was used to compare SRA ad SRN means attained in each NAM population.

Genome-wide association mapping analysis

Owing to the unbalanced design of the MR-NAM population, the small size of the NAM families, and the moderate selection pressure imposed for flowering time and height genes, the standard mapping approaches typically applied to NAM populations or bi-parental crosses were not appropriate. Here, we applied a multi-populational genomic regional QTL (mpQTL) analysis, a two-step process originally developed by Mace et al. (2013) in a sorghum backcross NAM population to overcome these limitations. For each marker, a P-

value testing the hypothesis that the two marker alleles have equal effect within each NAM family is generated. Fisher's combined probability test is then applied to combine the results of the single marker regression across all the NAM families to generate a single probability value. Here, we extended this approach to the MR-NAM population, by conducting single marker analysis within each NAM population instead of NAM families, and combining the results across NAM populations instead of NAM families.

The first step of the mpQTL involved single marker analysis within each of the three NAM populations. As polymorphic markers were unbalanced across NAM families (Appendix 2), an individual subset of markers was selected for each NAM population. This subset was obtained by selecting all the filtered markers from the different families that composed a NAM population. In total, 14,341 SilicoDArT markers were selected for Ma-NAM, 16,382 for Sc-NAM, and 16,916 for Su-NAM. In total, Ma-NAM and Sc-NAM shared 12,357 SilicoDArT, Ma-NAM and Su-NAM 12,652, and Sc-Nam and Su-Nam 14,881.

A genome-wide association study (GWAS) was performed within each specific NAM population using a single locus mixed model to control for population structure and relatedness, as described by Henderson (1975):

$$y_{ij} = \mu + f_i + \beta m_{ij} + g_{ij} + e_{ij}$$

where, y_{ij} is the phenotypic value (BLUEs) for jth individual in the ith family, μ is a general mean, f_i is the fixed effect of ith NAM family, β is the single marker fixed coefficient while m_{ij} is the single marker genotype for jth individual in the ith family, g_{ij} is the random polygenic effect of for jth individual in the ith family $g = (g_{ij})$~N(0,$\sigma_g^2 K$) and e_{ij} is the residual term e_{ij}~N(0,σ_e^2).

The kinship matrix was derived from all filtered markers except those on the chromosomes being tested. This helps to avoid loss of power due to the fact that markers are used for both testing association and for estimating relatedness (Rincent *et al.*, 2014).

GWAS was performed using the mixed linear model function implemented in TASSEL 5.2.33 (Bradbury *et al.*, 2007). GWAS was conducted for both seminal root traits (SRA and SRN), for each of the three NAM populations (Ma-NAM, Sc-NAM, and Su-NAM), and for each of the 21 chromosomes, resulting in 126 separate analyses. Marker effects were estimated in TASSEL and were expressed as the effect of the reference parent for each of the three NAM populations. A table of P-values for marker by NAM population was generated. To combine

the results of the single marker analysis across NAM population, the most significant marker was selected from a sliding window of length 5 cM and a step of 1 cM (Mace et al., 2013). This stepwise process was performed on each NAM population separately to generate a series of probability values spaced 1 cM apart along each chromosome. Fisher's combined probability test was then applied to combine the results of the single marker regression across the three NAM populations to generate a single probability value representing the approximate presence of significance at each 1 cM point along each chromosome. In order to overcome the imbalance across the NAM populations at each marker location, a false discovery rate adjustment was made to the Fisher P values to allow a consistent 0.01 % significance value (−log Fisher P value of >3) to declare QTL significance. Co-location of root trait QTL

Previously reported QTL for traits related to root system architecture in wheat were collated from three published studies (Hamada *et al.*, 2012; Christopher *et al.*, 2013; Maccaferri *et al.*, 2016). In total, 77 QTL were reported, including 34 QTL for SRA (Christopher *et al.*, 2013; Maccaferri *et al.*, 2016), 39 QTL for SRN (Hamada *et al.*, 2012; Christopher *et al.*, 2013; Maccaferri *et al.*, 2016), and four QTL related to gravitropic responses of wheat root (Hamada *et al.*, 2012). QTL identified by Christopher *et al*. (2013) were reassigned from a previous map using an older DArT marker system using the latest wheat DArT consensus map (http://www.diversityarrays.com/).

The location of individual QTL were projected onto the DArT consensus map along with the QTL identified in this chapter and the 13 hotspots identified in Chapter 4. A projection strategy using the SNP-based consensus map of tetraploid wheat as a bridge (Maccaferri *et al.*, 2015) was followed (Mace and Jordan, 2011). A confidence interval of 5 cM (i.e. 2.5 cM above and below the peak marker location) was implemented for display purposes. The DArT consensus marker data and QTL positions were visually displayed using Map-Chart v2.3 (Voorrips, 2002). Known, key agronomic genes, including dwarfing genes (Rht-B1 and Rht-D1) and phenology genes (Vrn-A1, Vrn-B1, Vrn-D1, Vrn-A3, Ppd-A1, and Ppd-B1) were also displayed on the map.

Results

Genetic diversity of the parental lines

The COP, which estimates the probability of two alleles in two different individuals being identical by descent, was globally low in the parental lines, with an average of 0.17 (Table 12). Dharwar Dry had less than 75% known parentage and was excluded from this analysis. The highest COP value was observed between SeriM82 and SB062 (0.67), as SeriM82 is a parent of SB062. Higher COP values were also observed by some parental lines sharing common ancestors in earlier generations (Table 9), for example Scout and Drysdale which share Hartog (0.48, Table 12).

Table 12: Coefficient of parentage for the three reference parents and ten out of the 11 founders used in this study to create the multi-reference parent nested association mapping population

Dharwar Dry was excluded from the analysis as it had less than 75% known parentage.

	Mace	***Scout***	***Suntop***	***Drysdale***	***EGA Gregory***	***EGA Wylie***	***RIL114***	***SB062***	***SeriM82***	***Westonia***	***ZWB10-37***	***ZWW10-128***
Scout	0.11											
Suntop	0.06	0.17										
Drysdale	0.07	0.48	0.23									
EGA Gregory	0.09	0.16	0.19	0.22								
EGA Wylie	0.12	0.12	0.13	0.16	0.23							
RIL114	0.08	0.14	0.16	0.18	0.50	0.18						
SB062	0.06	0.19	0.31	0.26	0.15	0.13	0.19					
SeriM82	0.06	0.20	0.39	0.29	0.15	0.11	0.15	0.67				
Westonia	0.12	0.11	0.12	0.13	0.12	0.11	0.10	0.12	0.12			
ZWB10-37	0.06	0.15	0.17	0.20	0.14	0.13	0.14	0.29	0.24	0.10		
ZWW10-128	0.06	0.21	0.19	0.29	0.16	0.14	0.16	0.27	0.26	0.12	0.20	
ZWW10-50	0.03	0.09	0.11	0.12	0.08	0.07	0.09	0.24	0.16	0.06	0.13	0.13

PCA based on SilicoDArT markers of the parental lines discriminated the references and the founders (Figure 17). Many lines developed at CIMMYT (e.g. SB062, SeriM82, ZWB10-37, ZWW10-50), were clustered together, close to the two Australian cultivars Drysdale and Scout, which both have considerable CIMMYT ancestry (Figure 17 and Table 9).

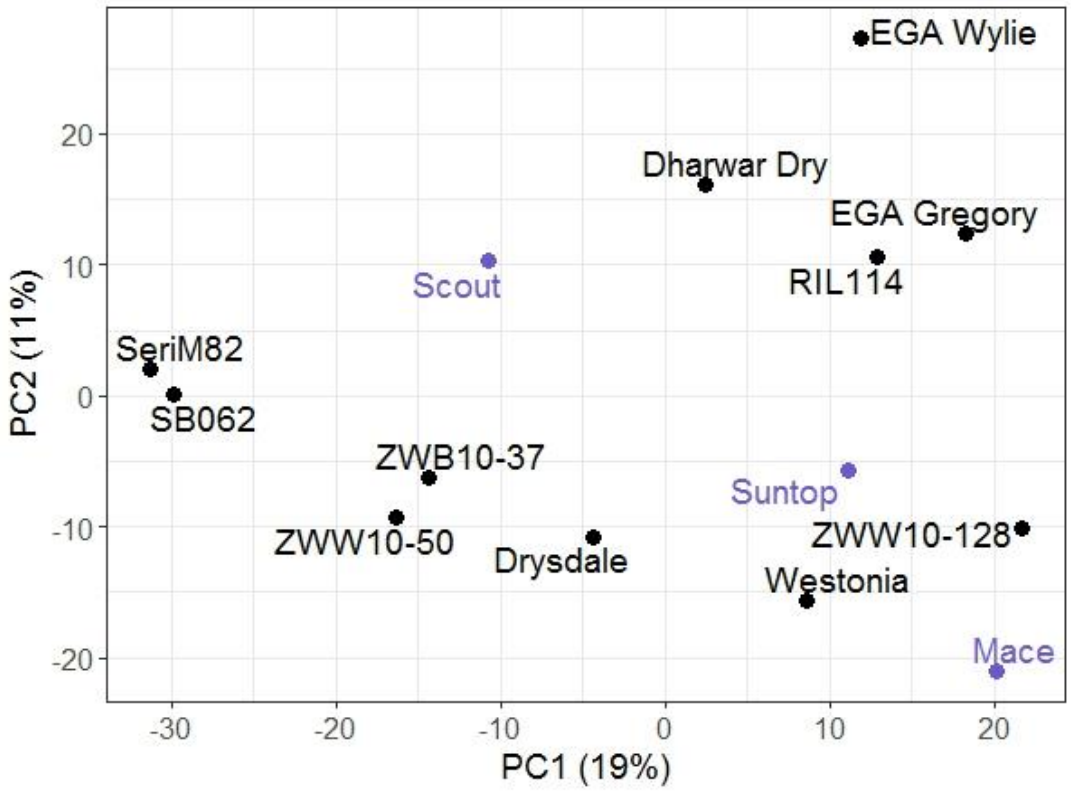

Figure 17: Biplot of the two first principal components from a principal component analysis of the genetic similarity for the 14 parental lines used to create the multi-reference parent nested association mapping population.

The two first axes represent 30% of the variation found within founders (black) and reference parents (purple).

Structure of the MR-NAM population

PCA based on filtered markers clearly differentiated the NAM lines into groups corresponding to each of the three reference parents (Figure 18).

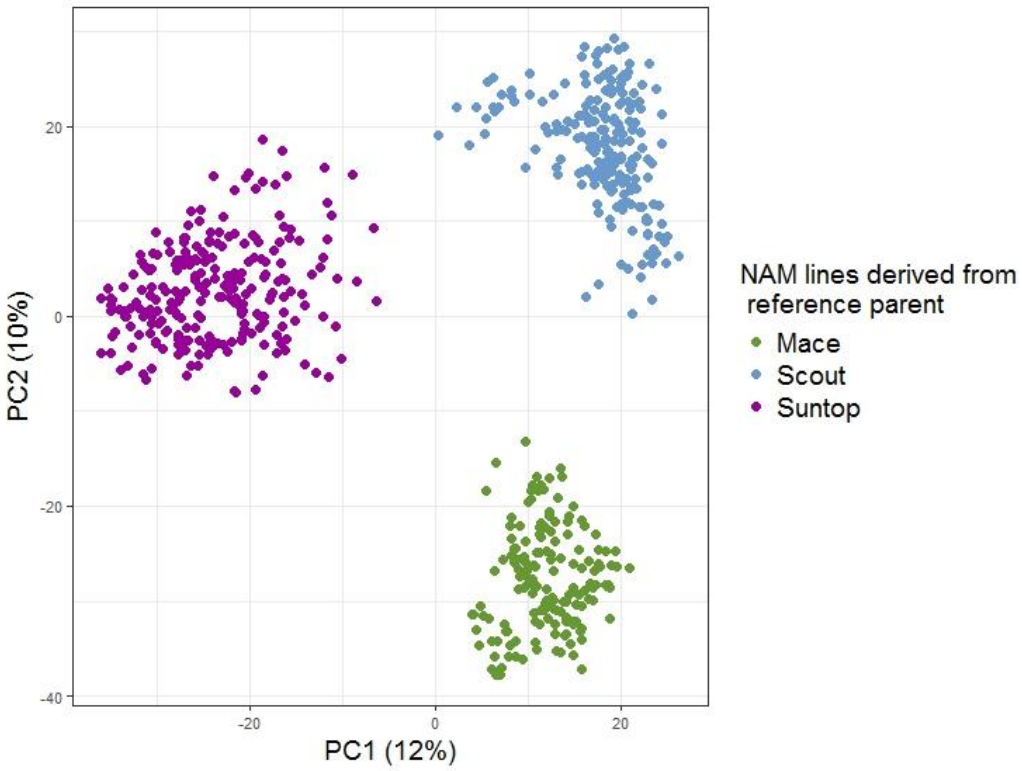

Figure 18: Biplot of the first two principal components from principal component analysis of the genetic similarity for 612 $F_{4:5}$ lines of the multi-reference parent nested association mapping population

PCA based on filtered markers discriminated all families in Ma-NAM and Sc-NAM (Figure 19). In Su-NAM, lines derived from founders EGA Gregory and EGA Wylie were less well separated, likely due to ancestors in common of these cultivars derived from the QDAF breeding program (Table 9). Similarly, lines from CIMMYT-derived lines SB062, ZWB10-37, and ZWW10-128 were also less well differentiated (Figure 19). Interestingly, SB062 and related parent SeriM82 were differentiated in Sc-NAM (Figure 19).

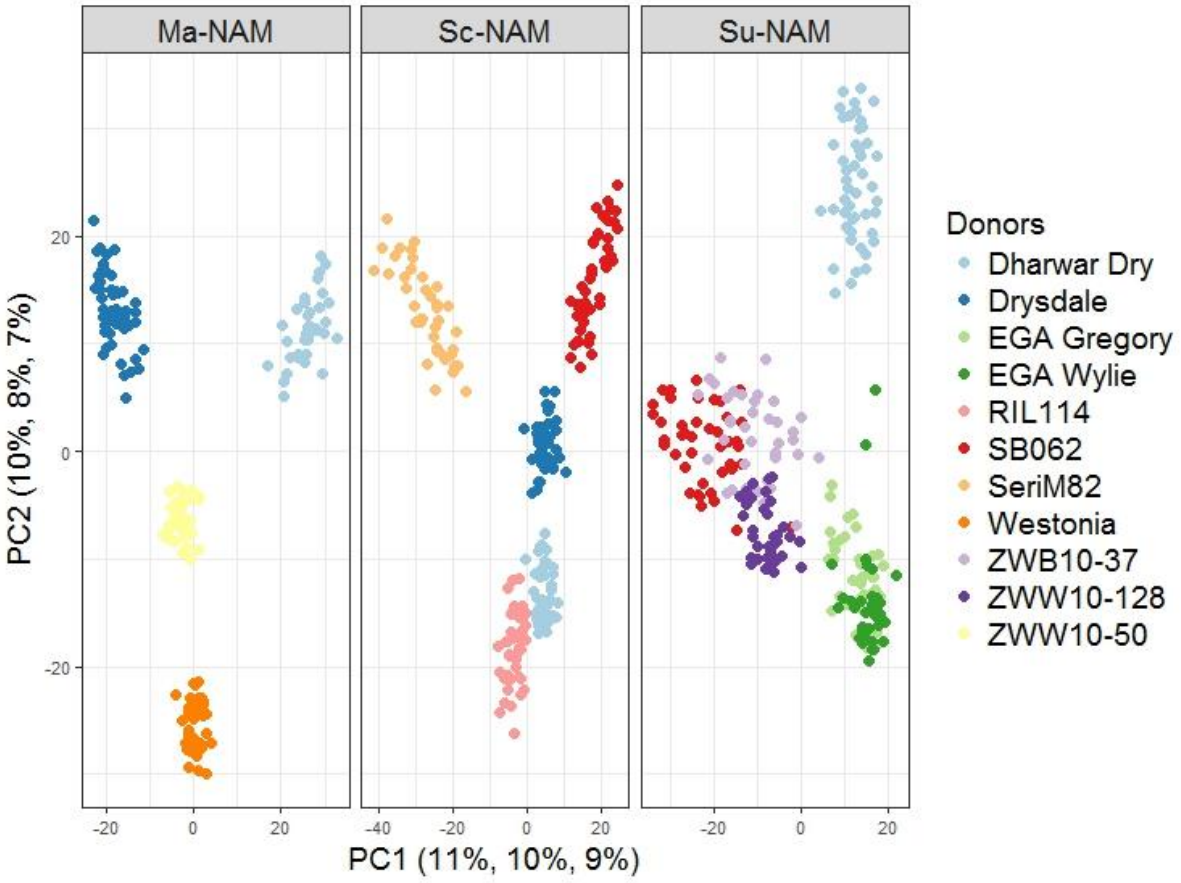

Figure 19: Biplot of the first two principal components from a principal components analysis of the genetic similarity for the 15 families derived from the three reference parents Mace (Ma-NAM), Scout (Sc-NAM), and Suntop (Su-NAM)

Marker data in the MR-NAM population

The lowest marker coverage was observed for chromosome 4D with 0.6 markers per cM, and the highest marker coverage was observed for chromosome 2B with 7.1 markers per cM (Table 11). Families Scout/RIL114 and Scout/SB062 had the lowest number of polymorphic markers in common (2,624), while Suntop/SB062 and Suntop/ZWB10-37 shared the highest number of polymorphic markers (5,940, Appendix 2). Markers were generally fairly evenly distributed on the consensus map for most chromosomes (Appendix 3).

LD decay in the MR-NAM population

Investigation of LD chromosome by chromosome showed a rapid decay with distance in each NAM population, except on chromosomes 1B and 2D for Sc-NAM and Su-NAM populations, respectively (Figure 20). LD decay averaged 6.6, 2.3, and 3.7 cM at a threshold $r^2 = 0.2$, and 1.5, 0.3, and 0.6 cM at a threshold $r^2 = 0.3$ (data not shown).

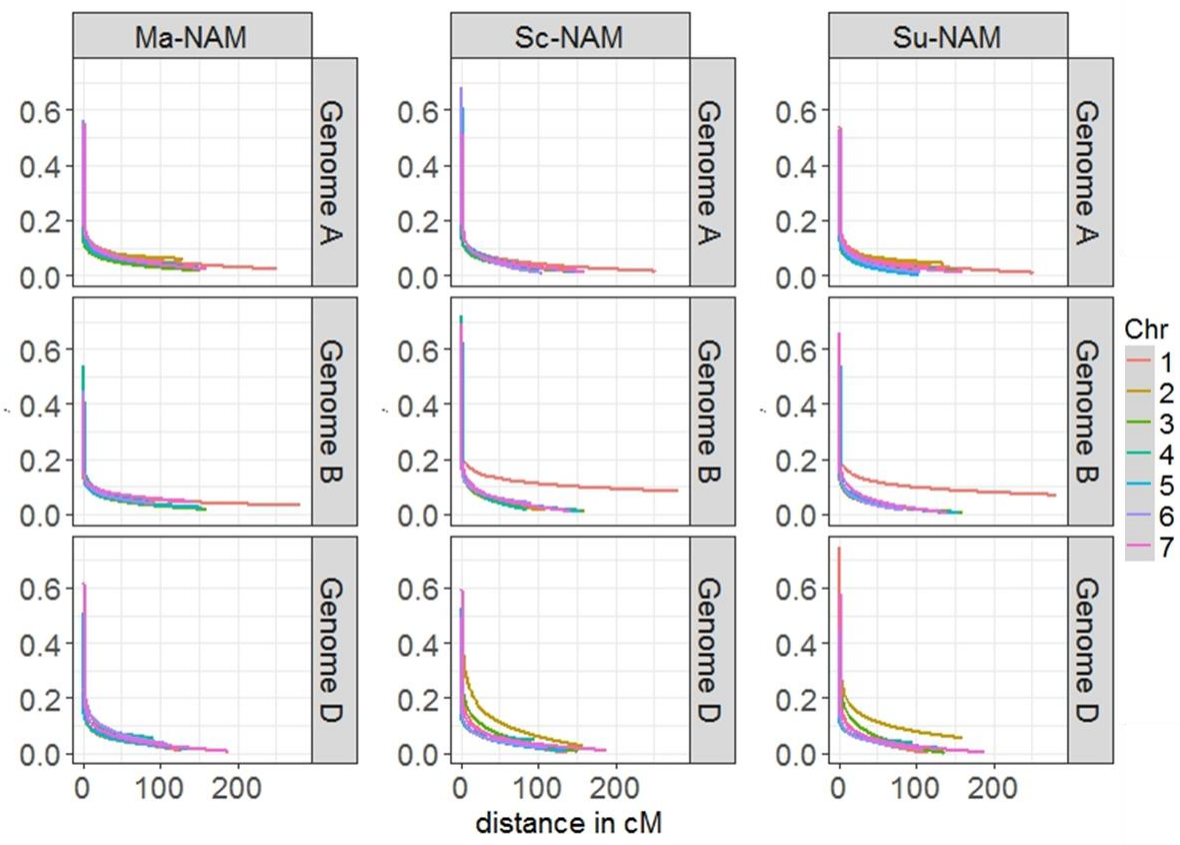

Figure 20: Linkage disequilibrium decay patterns for all chromosomes in each related nested association mapping population derived from Mace (Ma-NAM), Scout (Sc-NAM), and Suntop (Su-NAM).

Phenotypic variation for seminal root traits

Both root traits displayed high heritability (i.e. $h^2 = 0.63$ for SRA and $h^2 = 0.70$ for SRN) and high genetic variation within and across families (Figure 21). The smallest SRA was observed in a Scout/RIL114 family (41° ± 9°) and the highest in a Mace/Dharwar Dry family (114° ± 9°), corresponding to a range of 73° for SRA in the MR-NAM population (Figure 21). The smallest SRN was observed in a Scout/Drysdale family (5.7 ± 0.4) and the highest in a Scout/RIL114 family (10.0 ± 0.4), corresponding to a range in SRN of 4.3 for SRN in the MR-NAM population (Figure 21). SRA ranged from 61° (± 6°) to 114° (± 9°) in Ma-NAM (i.e. a range of 53°), from 41° (± 9°) to 100° (± 8°) in Sc-NAM (i.e. a range of 59°), and from 50° (± 7°) to 105° (± 7°) in Su-NAM (i.e. a range of 55°, Figure 21). SRN ranged from 4.8 (± 0.4) to 9.2 (± 0.4) in Ma-NAM (i.e. a range of 4.4), from 4.8 (± 0.4) to 10 (± 0.4) in Sc-NAM (i.e. a range of 5.2), and from 4.8 (± 0.4) to 8.3 (± 0.4) in Su-NAM (i.e. a range of 3.4, Figure 21).

When considering mean values for each of the three NAM, SRA was significantly higher (p-value < 0.01) in Ma-NAM (84° ± 9°) compared to Su-NAM (78° ± 10°) and Sc-NAM (69° ± 10°). SRN was also significantly higher (p-value < 0.01) in Su-NAM compared to Sc-NAM. SRN was significantly lower (p-value < 0.01) in Su-NAM (6.2 ± 0.62) compared to Ma-NAM

(6.5 ± 0.7) and Sc-NAM (6.5 ± 0.7), but not significantly different between Ma-NAM and Sc-NAM. SRA and SRN were significantly but weakly negatively correlated (r = -0.23, p-value < 0.01) in the MR-NAM population.

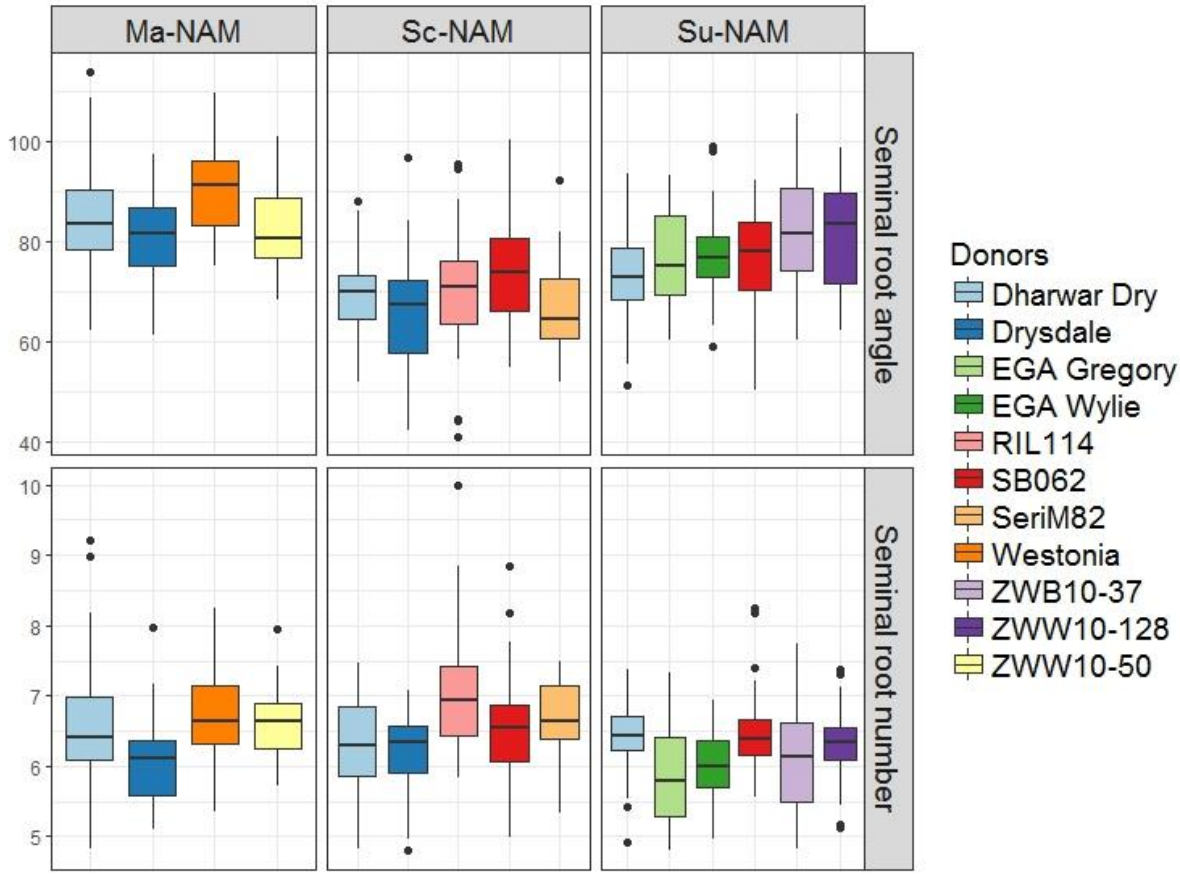

Figure 21: Distribution of estimated values for seminal root angle (top) and number (bottom) in a panel of 612 $F_{4:5}$ lines comprising 15 families in the multi-reference parent nested-association mapping population

The boxplots are grouped by families that share the same reference-parent, i.e. Mace-derived families (Ma-NAM), Scout-derived families (Sc-NAM), and Suntop-derived families (Su-NAM). The bottom and the top of the boxes display the first and third quartile values for each family, respectively. The band inside the box displays the median. The ends of the whiskers display the lowest and highest datum within 1.5 interquartile range of the lower and higher quartile, respectively. The outliers are represented as individual points. The values represent the best linear unbiased estimates (BLUEs).

Identification of QTL

A total of 29 QTL were identified in the MR-NAM population including 18 QTL for SRA on chromosomes 2A, 2B, chromosomes 2A, 2B, 3B, 5A, 5B, 6A, 7A, and 7B (

Table 13), and 11 QTL for SRN on chromosomes 2A, 2B, 2D, 3D, 4A, 5A, 6A, 6D, 7B, and 7D (Table 14).

Table 13: Summary of quantitative trait loci for seminal root angle identified in the multi-parent reference nested association mapping population

QTL were identified by combining the results of the single marker analysis across nested association mapping (NAM) populations derived from reference parents Mace (MA-NAM), Scout (Sc-NAM), and Suntop (Su-NAM) for each chromosome (Ch.).

Ch.	QTL ID[1]	Range (cM)	$-\log_{10}$ Fisher P at peak	Population	Peak Marker	Position at peak (cM)	$-\log_{10}$ P at peak	Effect at peak[2]
2A	QSra.qwr-2A.1	67.3 – 77.0	3.7	Ma-NAM	991548	69.5	3.5	-11 (20%)
				Sc-NAM	4004105	77.0	1.8	4 (7%)
				Su-NAM	993667	73.9	3.1	6 (11%)
	QSra.qwr-2A.2	119.8 - 123.7	3.8	Ma-NAM	1092017	120.7	3.3	-6 (11%)
				Sc-NAM	1129919	119.8	1.9	4 (7%)
				Su-NAM	2289207	123.7	3.0	6 (12%)
2B	QSra.qwr-2B.1	7.3 - 13.5	3.9	Ma-NAM	3022023	12.4	1.5	-6 (11%)
				Sc-NAM	5332999	12.6	2.6	-5 (8%)
				Su-NAM	1056599	12.4	4.2	8 (14%)
	QSra.qwr-2B.2	61.4 - 64.4	3.4	Ma-NAM	5360633	62.9	2.5	-9 (17%)
				Sc-NAM	5360633	62.9	2.3	7 (12%)
				Su-NAM	1111795	61.4	2.4	-6 (11%)
	QSra.qwr-2B.3	74.1 - 80.1	3.4	Ma-NAM	1865607	77.4	2.6	-5 (10%)
				Sc-NAM	2276879	78.3	2.1	6 (11%)
				Su-NAM	2257731	74.1	2.7	-6 (10%)
3B	QSra.qwr-3B.1	32.5 - 35.5	3.2	Ma-NAM	982680	32.5	1.6	-5 (9%)
				Sc-NAM	1056730	34.5	2.2	-6 (10%)
				Su-NAM	3936539	35.5	3.0	-6 (11%)
	QSra.qwr-3B.2	64.9 - 67.9	3.4	Ma-NAM	982093	67.5	1.4	-7 (12%)
				Sc-NAM	2282368	64.9	3.1	-8 (14%)
				Su-NAM	1318762	65.0	2.9	6 (11%)
5A	QSra.qwr-5A.1	13.2 - 23.8	3.9	Ma-NAM	1102789	23.8	2.7	8 (16%)
				Sc-NAM	1141722	14.5	4.5	-14 (23%)
				Su-NAM	1141722	14.5	1.6	-3 (6%)
	QSra.qwr-5A.2	36.3 – 40.0	4.6	Ma-NAM	1008358	36.9	3.1	10 (18%)
				Sc-NAM	5410407	36.9	4.7	-13 (22%)
				Su-NAM	4261703	36.3	2.1	-4 (7%)
	QSra.qwr-5A.3	84.9 - 89.7	3.2	Ma-NAM	1119105	84.9	2.3	-5 (10%)
				Sc-NAM	1094285	86.7	3.1	-8 (13%)
				Su-NAM	1218026	86.2	1.6	3 (6%)
5B	QSra.qwr-5B.1	32.8 - 36.6	3.2	Ma-NAM	1097023	33.3	1.8	7 (14%)
				Sc-NAM	1108302	32.8	2.6	-6 (10%)
				Su-NAM	1150272	36.6	2.4	-7 (13%)
	QSra.qwr-5B.2	60.1 - 61.7	3.0	Ma-NAM	1254570	60.1	1.5	4 (7%)
				Sc-NAM	1042425	61.7	2.3	-8 (13%)
				Su-NAM	1107344	60.1	2.6	-9 (16%)
6A	QSra.qwr-6A.1	42.0 - 60.9	3.9	Ma-NAM	1126827	43.2	3.6	11 (21%)

				Sc-NAM	1127951	43.0	2.7	-7 (12%)
				Su-NAM	2276793	47.6	3.3	-11 (21%)
	QSra.qwr-6A.2	78.6 - 99.5	3.9	Ma-NAM	3026160	78.6	3.4	6 (12%)
				Sc-NAM	1104624	95.9	3.3	-6 (11%)
				Su-NAM	3024279	98.8	3.2	-11 (20%)
6B	QSra.qwr-6B.1	14.1 - 22.6	3.4	Ma-NAM	1696386	14.1	1.5	-5 (9%)
				Sc-NAM	1102638	21.3	2.4	9 (14%)
				Su-NAM	4543610	18.4	4.3	-6 (11%)
	QSra.qwr-6B.2	78.3 - 82.2	3.8	Ma-NAM	4405558	79.2	1.7	5 (9%)
				Sc-NAM	1106105	81.7	3.3	8 (13%)
				Su-NAM	1095414	78.4	3.1	4 (8%)
7A	QSra.qwr-7A	97.6 - 103.1	3.4	Ma-NAM	1106114	99.3	2.1	-5 (10%)
				Sc-NAM	1216850	101.5	3.0	14 (24%)
				Su-NAM	3533452	97.6	2.0	-7 (13%)
7B	QSra.qwr-7B	102.2 - 106.9	3.1	Ma-NAM	1267473	102.2	1.4	5 (10%)
				Sc-NAM	1147335	105.3	3.4	-6 (11%)
				Su-NAM	1043428	106.9	1.8	-4 (8%)

[1] QTL were named according to McIntosh Catalogue of Gene Symbols for Wheat (http://wheat.pw.usda.gov/ggpages/wgc/98/). Sra is used to represent seminal root angle, qwr for Queensland Alliance for Agriculture and Food Innovation Wheat Research, followed by chromosome number and an Arabic numeral.

[2] Seminal root angle (SRA) in degrees. The value in bracket indicates the percentage of the total SRA variation explained by the QTL for the corresponding NAM population. Positive values (+) indicate that the allele from the reference parent contributes wider SRA while negative values (-) indicate narrower SRA.

Table 14: Summary of quantitative trait loci for seminal root number identified in the multi-parent reference nested association mapping population

QTL were identified by combining the results of the single marker analysis across nested association mapping (NAM) populations derived from reference parents Mace (MA-NAM), Scout (Sc-NAM), and Suntop (Su-NAM) for each chromosome (Ch.).

Ch.	QTL ID[1]	Range (cM)	$-\log_{10}$ Fisher P at peak	Population	Peak Marker	Position at peak (cM)	$-\log_{10}$ P at peak	Effect at peak[2]
2A	QSrn.qwr-2A	120.7 - 123.7	3.5	Ma-NAM	4404868	120.7	3.3	-0.5 (11%)
				Sc-NAM	1666222	123.7	2.6	-0.8 (15%)
				Su-NAM	1241953	123.7	2.1	-0.3 (8%)
2B	QSrn.qwr-2B.1	28.3 - 34.1	3.1	Ma-NAM	5969694	30.5	2.6	0.8 (18%)
				Sc-NAM	3064507	28.7	2.6	-0.7 (13%)
				Su-NAM	1035323	34.1	2.5	0.3 (8%)
	QSrn.qwr-2B.2	61.5 - 63.7	3	Ma-NAM	1370909	63.7	2.2	-0.6 (15%)
				Sc-NAM	5360633	62.9	2.6	-0.5 (10%)
				Su-NAM	2276283	61.5	2.2	0.4 (12%)
2D	QSrn.qwr-2D	126.8 - 129.7	3.3	Ma-NAM	4018191	129.7	2.5	0.5 (11%)
				Sc-NAM	5332356	126.8	2.7	-0.5 (10%)
				Su-NAM	3953789	128.5	2.6	0.2 (7%)
3D	QSrn.qwr-3D	146.6 - 149.3	3.3	Ma-NAM	1074810	149.3	2.8	-0.6 (13%)
				Sc-NAM	1087824	147.1	3.2	-0.7 (14%)

				Su-NAM	1219057	146.6	1.7	0.4 (11%)
4A	QSrn.qwr-4A	102.3 - 103.7	3	Ma-NAM	3947495	103.7	1.2	0.5 (11%)
				Sc-NAM	1014798	103.4	4.4	-0.8 (16%)
				Su-NAM	1298634	102.3	1.4	-0.2 (6%)
5A	QSrn.qwr-5A	36.2 - 50.1	3.8	Ma-NAM	1230744	49.4	3.5	-0.9 (21%)
				Sc-NAM	1293026	36.6	4.2	-0.9 (17%)
				Su-NAM	4541502	39.3	2.9	-0.6 (16%)
6A	QSrn.qwr-6A	94.1 - 99.2	3.8	Ma-NAM	1386046	94.1	2.4	0.3 (8%)
				Sc-NAM	1234105	98.9	2.9	-0.5 (10%)
				Su-NAM	3960864	98.8	3.4	-0.3 (10%)
6D	QSrn.qwr-6D	46.4 - 48.6	3.3	Ma-NAM	1250659	46.4	2.2	-0.4 (10%)
				Sc-NAM	3941820	47.2	3.8	-0.6 (11%)
				Su-NAM	3941820	47.2	1.7	-0.3 (9%)
7B	QSrn.qwr-7B	100 - 104.8	3.3	Ma-NAM	3948451	101.5	2.9	-0.8 (18%)
				Sc-NAM	3948451	101.5	2.2	-0.6 (11%)
				Su-NAM	1248026	100	2.4	-0.5 (15%)
7D	QSrn.qwr-7D	32.8 - 36.7	3.6	Ma-NAM	2251698	34.3	0.9	-0.3 (7%)
				Sc-NAM	1108038	36.1	5	-1 (18%)
				Su-NAM	1108038	36.1	2.4	0.5 (16%)

[1] QTL were named according to McIntosh Catalogue of Gene Symbols for Wheat (http://wheat.pw.usda.gov/ggpages/wgc/98/). Srn is used to represent seminal root number, qwr for Queensland Alliance for Agriculture and Food Innovation Wheat Research, followed by chromosome number and an Arabic numeral.

[2] Number of seminal roots. The value in bracket indicates the percentage of the total seminal root number (SRN) variation explained by the QTL for the corresponding NAM population. Positive values (+) indicate that the allele from the reference parent contributes higher SRN while negative values (-) indicate lower SRN.

Within each NAM population, QTL for both SRA and SRN donated by the reference parent had both negative and positive effects (

Table 13 and Table 14).The QTL with the largest effect for SRA in Ma-NAM, QSra.qwr-6A.1, had an estimated effect of +11°, which explained 21% of the total SRA range of 53° (

Table 13). The QTL with the least effect, QSra.qwr-5B.2, had an estimate effect of +4° (i.e. 7% of the total SRA range,

Table 13). In Sc-NAM, the QTL with the largest effect, QSra.qwr-7A, had an estimated effect of +14°, which explained 24% of the total SRA range of 59° (

Table 13). The QTL with the least effect, QSra.qwr-2A.1 and QSra.qwr-2A.2, had an estimated effect of +4° (i.e. 7% of the total SRA range,

Table 13). In Su-NAM, the QTL with the largest effect, QSra.qwr-6A.1, had an estimated effect of -11°, which explained 21% of the total SRA range of 55° (

Table 13). The QTL with the least effect, QSra.qwr-5A.1 and QSra.qwr-5A.3, had an estimate effect of -3° and +3°, respectively (i.e. 6% of the total SRA range,

Table 13). For SRN, the largest QTL in Ma-NAM, explained 21% of the range of 4.4 roots, the smallest explaining 7% of the range (Table 14). For Sc-NAM, the largest QTL for SRN explained 18% of the range of 5.2 roots and the largest for Su-NAM explained 16% of the range of 3.4 roots (Table 14). The smallest QTL for SRN explained 10% and 6% in Sc-NAM and Su-NAM respectively (Table 14).

The confidence intervals of QTL varied greatly from a single marker to large parts of chromosome segments (

Table 13, Table 14, and Appendix 3). Out of the 18 QTL identified for SRA, seven QTL collocated with previously reported genomic regions related to SRA (Appendix 3). On chromosome 2B and 6A, QSra.qwr-2B.2, and QSra.qwr-6A.2 collocated with hotspots identified in selected tails populations in Chapter 4 (*hp2*, and *hp12*, respectively), and two QTL identified in Christopher *et al.* (2013). On chromosome 2A, 2B, 5B, and 6B, QSra.qwr-2A.1, QSra.qwr-2A.2, QSra.qwr-2B.1, QSra.qwr-5B.1, and qSra.qwr-6B.2 collocated with three QTL identified in Maccaferri *et al.* (2016), a QTL identified in Christopher *et al.* (2013), and a QTL identified in (Hamada *et al.*, 2012). QTL QSra.qwr-2B.1, QSra.qwr-3B.1, QSra.qwr-6B.1, and QSra.qwr-7A were closely located (< 5 cM) to hp10, hp4, hp13, and hp7, respectively. Interestingly, out of the 13 hotspots identified in Chapter 4, five hotspots collocated with QTL identified in previous studies (Appendix 3). For SRN, out of the 11 QTL identified, two QTL, QSrn.qwr-2B2 and QSrn.qwr-5A, collocated with QTL for root number identified in Maccaferri *et al.* (2016) and Hamada *et al.* (2012). Thus in total, eleven of the QTL for SRA and nine QTL for SRN in the current study appear novel. Some QTL for SRA overlapped with some QTL for SRN, such as QSra.qwr-2A.2 and QSrn.qwr-2A on chromosome 2A, QSra.qwr-2B.2 and QSrn.qwr-2B.2 on chromosome 2B, QSrn.qwr-5A and QSra.qwr-5A.2 on chromosome 5A, QSra.qwr-6A.2 and QSrn.qwr-6A on chromosome 6A, and QSra.qwr-7A and QSrn.qwr-7A on chromosome 7A (

Table 13, Table 14, and Appendix 3).

Discussion

We believe this is the first report of a MR-NAM population that incorporates a large amount of diversity from a panel of founders into agronomically relevant reference parents in a cereal crop. This resource is suitable for both geneticists aiming to understand the genetic architecture of complex traits and for plant breeders aiming to develop improved cultivars in diverse environments. We used this powerful pre-breeding platform to investigate the genetic control of seminal root traits. The genetic diversity represented in this resource allowed us to identify novel QTL for both SRA and SRN. Results indicate that SRA and SRN

are governed by a large number of QTL with both minor and moderate effects, which varied depending on the genetic background.

Multiple QTL for seminal root traits were identified

By integrating analyses across the three NAM populations, we identified a total of 18 QTL for SRA and 11 QTL for SRN (

Table 13 and Table 14). Founders of the MR-NAM were selected foremost for a wide range of adaptive traits followed by genetic diversity to maximise allele richness across the panel. As a result, a high degree of variation was found for both seminal root traits. The range of SRA was 53, 55, and 59° in Ma-NAM, Sc-NAM, and Su-NAM, respectively, corresponding to a total range of 73° in the MR-NAM population. These ranges were greater than the range of 24° reported in Chapter 3, 34° reported in Chapter 4, and compared to previous studies (Manschadi *et al.*, 2008; Hamada *et al.*, 2012; Christopher *et al.*, 2013). The total additive effect was -63, -87, and -80° for all QTL alleles contributing to narrow SRA, and 57, 52 and 33° for all QTL alleles contributing to wide SRA originating from Mace, Scout, and Suntop, respectively. Interestingly, Mace and Suntop, which both displayed wide SRA (Figure 11), also contributed alleles for narrow SRA. This is consistent with what we found previously in Chapter 4. It also agrees with other previous reports (Hamada *et al.*, 2012; Christopher *et al.*, 2013; Maccaferri *et al.*, 2016). The range of SRN was 4.4, 5.2, and 3.4 in Ma-NAM, Sc-NAM, and Su-NAM, respectively, corresponding to a range of 5.2 in the MR-NAM population. These ranges were greater than the range of 1.3 reported in Chapter 3 where plants were grown under the same conditions for 11 days at 17°C , and compared to previous studies where plants were grown for eight days at 15°C (Manschadi *et al.*, 2008; Christopher *et al.*, 2013). However, these ranges were smaller than the range of 7 reported in a diverse wheat panel grown for seven days with day/night temperatures of 28/22°C (Hamada *et al.*, 2012). Such differences may be attributed to soil temperature before seedling emergence (Richards and Passioura, 1981). The total additive effect was -4.2, -7.6, and -2.2 for all QTL alleles contributing to low SRN, and 2.1, 0, and 1.8 for all QTL alleles contributing to high SRN originating from Mace, Scout, and Suntop, respectively.

The observation that each reference parent contributed alleles for both narrow and wide SRA and alleles for both low and high SRN is consistent with transgressive segregation and complex inheritance, as previously reported (Liu *et al.*, 2013; Christopher *et al.*, 2013). Each trait is controlled at multiple loci of small to moderate effect, with complex QTL – QTL interactions (Liu *et al.*, 2013; Christopher *et al.*, 2013). Moreover, some QTL for SRA and SRN overlapped on five different chromosomes. Thus, it is possible that a single gene could underpin both root traits within these genomic regions. This evidence for complex genetic control in wheat contrasts with previous reports in rice (*Oryza sativa* L.), where a single major gene, DEEP ROOTING 1 (*DRO1*), explained 66.6% of the total phenotypic variance in the ratio of deep rooting (Uga *et al.*, 2011). However, this QTL was identified in population derived from a single cross. Hence QTL effect may be smaller in a wider array of crosses because of possible epistatic interaction between QTL and genetic background.

Some QTL for seminal root traits were novel

In recent years, QTL have been reported in wheat for SRA, SRN, and related root architectural traits (Hamada *et al.*, 2012; Liu *et al.*, 2013; Bai *et al.*, 2013; Christopher *et al.*, 2013; Maccaferri *et al.*, 2016). Almost half of the QTL identified for SRA in this study co-located with previous reported wheat QTL, while only one QTL for SRN co-located with previous reported QTL. Interestingly, some hotspots for SRA identified in Chapter 4 were overlapping some QTL identified in this study and/or QTL identified in previous studies (Appendix 3). However, QTL for SRA QSra.qgw-2B.3, QTL QSra.qgw-3B.1, QSra.qgw-3B.2, QSra.qgw-5A1, QSra.qgw-5A.2, QSra.qgw-5A.3, QSra.qgw-5B.2, QSra.qgw-6A.1, QSra.qgw-6B.1, QSra.qgw-7A.1, and QSra.qgw-7B have not been previously identified in a published study to our knowledge and likely represent novel QTL. Similarly, QTL for SRN QSrn.qgw-2A, QSrn.qgw-2B.1, QSrn.qgw-2D, QSrn.qgw-3D, QSrn.qgw-4A, QSrn.qgw-6A, QSrn.qgw-6D, QSrn.qgw-7B, and QSrn.qgw-7D have not been previously identified in a published study to our knowledge and likely represent novel QTL. Due to the lower coverage on genome D, with some chromosomes having less than one marker per cM, additional QTL controlling both SRA and SRN might remain undetected using the current map. Furthermore, the moderate selection for height and flowering time, could also have fixed some regions and potentially affected QTL detection.

QTL for a range of traits related to root system architecture have been reported in other species (Comas *et al.*, 2013). In rice, *DRO1* is located on the long arm of rice chromosome 9 (Uga *et al.*, 2011). Comparative genetic studies in cereals (Gale and Devos, 1998)

indicated a syntenic relationship between rice chromosome 9 and wheat homoeologous group 5. In maize (*Zea mays* L.), a QTL related to root angle has been reported on chromosome 7 (Omori and Mano, 2007), which has a syntenic relationship with rice chromosome 9, and could be homoeologous to *DRO1* (Uga *et al.*, 2011). Further, a major QTL for SRA and SRN was identified in barley (*Hordeum vulgare* L.), on chromosome 5 (Robinson *et al.*, 2016). In this study, three QTL for SRA were located on chromosome 5A (*QSra.qgw-5A.1*, *QSra.qgw-5A.2* and *QSra.qgw-5A.3), and two on chromosome 5B (QSra.qgw-5B.1* and *QSra.qgw-5B.2)*. It is therefore, possible that there is a relationship between one or more of these QTL and *DRO1.* The wheat QTL, as might be anticipated, each have a much smaller effect (from 6 to 24%) than *DRO1* of rice (67%).

The effect of key developmental genes on seminal root traits requires further investigation

The genetic control of flowering time in wheat is complex (Slafer et al., 2001; Trevaskis et al., 2007). Control of phenology involves photoperiod and vernalisation responses as well as earliness *per se*. Phenology affecting genes can strongly impact yield and many other traits (Xiao *et al.*, 2017). Parental lines of the MR-NAM population presented a combination of different alleles for phenology genes, so that segregation of phenology genes was anticipated in some families. Here, three QTL for SRA and two QTL for SRN were identified on 2B where *Ppd-B1* is located (QSra.qwr-2B.1, QSra.qwr-2B.2, QSra.qwr-2B.3, QSrn.qwr-2B.1 and QSrn.qwr-2B.2). Two QTL were closely located to *Ppd-B1*, at 6 cM distance for SRN (QSrn.qwr-2B.1) and 20 cM for SRA (QSra.qwr-2B.2, Appendix 3). Two QTL for SRA and one QTL for SRN were identified on 2A where the photoperiod gene *Ppd-A1* is located (QSra.qwr-2A.1, QSra.qwr-2A.2, and qSrn.qwr-2A). However, these QTL were not very closely located, being minimum 42 cM *from Ppd-A1* (Appendix 3). Therefore, further studies are required to confirm any potential relationship between photoperiod genes and root traits.

Three QTL for SRA and one QTL for SRN were identified on 5A, where the vernalisation gene *Vrn-A1* is located (QSra.qwr-5A.1, QSra.qwr-5A.2, QSra.qwr-5A.3, and QSrn.qwr-5A). Parental lines were carrying different alleles for *Vrn-A1*, so it is likely that some families were segregating for this trait. QTL QSra.qwr-5A.3 aligned with *Vrn-A1*. Hence this QTL may be a pleiotropic effect from known *Vrn-A1* genes. Two QTL for SRA were identified on 5B but were not co-located with *Vrn-B1*. Similarly, on QTL for SRA was identified on 5D but was not co-located with *Vrn-A3*. No QTL were identified on chromosomes 5D, where gene *Vrn-D1*is located. Thus further studies are required to confirm any impact of major vernalisation

loci on seminal root traits. In a previous study of barley, some seminal root angle QTL coincided with vernalisation genes (Hamada *et al.*, 2012).

Semi-dwarfing genes reduce lodging, increase yield in some environments, and can also impact many physiological and morphological traits in wheat (Rebetzke *et al.*, 2012*a*). Parental lines used in this study presented alleles for either Rht-B1b or Rht-D1b. Therefore some families segregating for Rht-B1b and Rht-D1b were anticipated in the MR-NAM population. However, no QTL were detected on 4B nor 4D, suggesting that there is no genetic relationship between Rht genes and the identified QTL controlling root traits. In previous studies, some QTL for a range of seedling root traits were coincident with dwarfing loci in wheat (Bai *et al.*, 2013).

The MR-NAM population provides a powerful tool to detect QTL

Many QTL for agriculturally relevant traits have been identified in bi-parental populations obtained by crossing two parental lines divergent for the trait of interest (Kearsey and Farquhar, 1998). The bi-allelic nature of these populations can pose a constraint by restricting the number of polymorphic loci and only allowing two alleles to be studied at each locus. The MR-NAM population developed in this study offers opportunity to assess the effect of multiple alleles at a locus and at the same time to investigate interactions between loci. Some markers were polymorphic in only one or few NAM families, suggesting that some regions were still segregating in some NAM families. In the current study, we elected to maximise the number of NAM families in order to maximise genetic diversity with smaller family sizes ranging from 34 to 50 RILs per NAM family. Although a small family size decreases the power to detect QTL within a single NAM family, the power of the MR-NAM resource comes from integrating analyses across families and references.

The NAM approach allows the use of an appropriate controlled crossing structure (Yu *et al.*, 2008). Spurious associations may arise when population structure is not correctly controlled for, while excessive control can lower the power to detect QTL (Larsson *et al.*, 2013). PCA showed distinct clusters of the families within each NAM population, except in the Su-NAM, where multiple founders deriving from the same breeding program were included in the crossing design. This population stratification was included in our association mapping to model families as a fixed intercept, and kinship to control for relatedness among individuals.

Within chromosomes, LD decay displayed similar patterns, except on chromosomes 1B and 2D, likely due to errors ordering markers in the consensus map. LD was found to decay to the threshold value of $r^2 = 0.2$ at less than 6.6 cM on average in the three NAM populations, which is similar to the LD decay of 9.2 cM reported in a barley advanced backcross NAM population (Nice *et al.*, 2016). LD was found to decay to the threshold value of $r^2 = 0.3$ at less than 1.5 cM on average in the three NAM populations. Mapping resolution is thus improved compared to many other recombinant inbred populations where confidence intervals are mostly within 10 – 20 cM (Abdurakhmonov and Abdukarimov, 2008).

The high average mapping density of polymorphic markers in the MR-NAM population (2.6 markers per cM) allows for nearly complete genome coverage and high confidence in QTL identification. The coverage was unequally distributed with the lowest coverage on the D genome, which is consistent with previous studies in wheat where the D genome has been reported to have much lower marker coverage than the A and B genomes (Akhunov *et al.*, 2010). The D genome has lost a large fraction of the diversity found in the progenitor genome of *Aegilops tauschii* (Caldwell *et al.*, 2004). Hence, this reduced diversity in the D genome of *T. aestivum* cultivars is a limitation to genetic map construction (Cavanagh *et al.*, 2013).

The MR-NAM population captures high genetic diversity

Founders of the MR-NAM population were selected based on the likelihood that they would produce new genetic combinations relevant for diverse environments such as encountered in the Australian wheat belt. In addition to root traits, parents and founders were selected for variation in drought and heat adaptation, stay-green traits, multiple disease resistance, acid soil tolerance, and pre-harvest sprouting tolerance. Pedigree was also considered to maximise genetic diversity. The 11 founders originated from six different breeding programs in a range of countries including Mexico, India, and Australia. The average COP of 0.17 for parental lines was slightly higher than the COP reported by Barbosa Neto (1995) in a panel of 31 diverse wheat cultivars (COP = 0.10) and by Bered *et al.* (2002) among 53 wheat inbred lines and cultivars used in genetic improvement programs in the south of Brazil (COP = 0.07). PCA based on SilicoDArT markers gave clustering groups slightly different than those obtained using COP values. The observation of differences between COP and genetic dissimilarities based on DNA markers is consistent with some previous studies (Martin *et al.*, 1995; Barbosa-Neto *et al.*, 1996; Bohn *et al.*, 1999; Corbellini *et al.*, 2002). This is because calculations for COP rely on available pedigree information sometimes limited for breeding material, and do not consider selection history nor random effects (Corbellini *et al.*,

2002). Despite these discrepancies, both pedigree and molecular data indicated a high level of genetic diversity among the founders.

The cultivar Dharwar Dry was selected by CIMMYT breeders for its high tolerance to drought in central India (Kirigwi *et al.*, 2007; Alexander *et al.*, 2012). The pedigree of Dharwar Dry could not be related to any breeding programs, but it has been speculated that it may be partly derived from CIMMYT germplasm (Kirigwi *et al.*, 2007). However, in the current study, Dharwar Dry was genetically closer to some Australian cultivars than the CIMMYT lines used in this study. Therefore, the abiotic tolerance genes in Dharwar Dry may be different to those carried by CIMMYT lines because they are likely to be relatively unrelated.

Opportunities for wheat breeding

The novel MR-NAM system developed here offers the advantages of studying genetics in diverse environments while breeding for adaptation to them at the same time. The use of the chosen elite cultivars has several benefits. The three reference parents Suntop, Scout and Mace, are relevant to each of the three major Australian cropping regions; the east, south and west, respectively. Crops growing in each of these regions experience widely different climatic conditions. The Australian wheat belt presents highly diverse rainfall patterns and soil types across regions. The western mega-region has winter dominant and sandy soils with low water holding capacity so mainly in-crop rainfall. The southern region ranges from near uniform winter rainfall to winter dominated rainfall, with variable soil types in terms of water-holding capacity. The eastern mega region of Australia has summer dominant rainfall so that winter grown wheat crops rely heavily on moisture stored in deep clay soils from rainfall the previous summer. Thus, the Australian mega-regions each present crop moisture stress patterns that are similar to different regions in other parts of the globe (Hodson and White, 2007). By using three reference parents adapted to different mega-regions, we developed three populations, each targeting different environment types.

As opposed to some other mapping populations, the genetic background of each of the three NAM is already suitable for the breeding target environments. Therefore, the derived lines can be used directly in a breeding program, either for selection or for relevant marker-trait associations. This reduces the number of breeding cycles in the process of gene transfer into adapted material, and subsequently a cultivar. Furthermore, the effect of the introgressed allele may vary because of epistatic effects. For example, the breeding value of an allele is dependent on the genetic backgrounds in which it is evaluated. Therefore the

use of relevant elite genetic backgrounds allows identification of favourable genotypes or alleles for selection within an applied breeding program. By including a generation of selection in the field, we produced $F_{4:5}$ NAM lines with height and flowering time adapted to the different regions in Australia. In addition, extreme phenotypes such as double dwarf and late maturing lines were eliminated during the RIL development. By selecting plants with similar height and flowering time, we reduced confounding effects, and discarded material mal-adapted for breeding purposes.

NAM populations increase genetic diversity and recombination events to facilitate further reductions in LD so that even minor effect QTL can be identified (Yu *et al.*, 2008). Compared to NAM populations, MAGIC populations typically give rise to high levels of recombination due to the multiple cycles of intercrossing between multiple founders, and hence greater precision in QTL location (Kover et al., 2009). However MAGIC populations take more time and work to create because of the number of generations required to intercross all the parental lines (Mackay et al., 2014). For example, it requires three generations to obtain 8-way inter-crosses to create an eight-parent MAGIC population before developing the RILs by single seed descent. Once developed, MAGIC populations are fixed and the population cannot be expanded by adding new parental lines to increase the genetic diversity. NAM populations are generally less powerful than MAGIC populations due to the common parent strategy employed. However, NAM populations are better adapted to facilitate evaluation in the field (Yu et al., 2008). For example, it is easier to compare lines with a reduced flowering time window when studying drought, as any variations for this trait would bring forward or postpone the key developmental stages, causing different types of drought stress. In addition, as NAM populations can rapidly be expanded, new parental lines can be added to the population to better suit the breeders' current priorities, while increasing the diversity and the power of the population.

Conclusions

Here, we report the development and utilisation of, we believe, the first wheat MR-NAM population, which provides a powerful and evolving tool to identify QTL in agriculturally relevant material for breeding programs. We report novel QTL for seminal root traits. From a breeding perspective, the study presented here provides a valuable estimate of the effects of QTL alleles across three elite genetic backgrounds adapted to each of the three major

regions of the Australian wheat belt. These QTL are promising candidates for positional cloning which would eventually allow development of ‘perfect markers’ to improve the efficiency of marker-assisted breeding strategies.

The MR-NAM strategy provides an optimum framework for an evolving pre-breeding platform that can be expanded over time to facilitate genetic analysis and introgression of mapped QTL into elite germplasm. We anticipate such platforms will be further enhanced by the application of high-throughput phenomic platforms (Gegas et al., 2014). Layering of phenotypic data sets will allow comparison of QTL interactions across multiple traits to help to understand epistasis, additive, synergistic and/or antagonist effects.

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Chapter 6
General discussion

Introduction: an outline of the fulfilment of objectives

A major challenge for agriculture is to meet the growing demand for food, feed, and fuel in the face of limited arable land and climate change**.** Water-limitation is one of the major constraints for production of wheat (*Triticum aestivum* L.), which is a major source of nutrition in many parts of the world. The aim of this body of research was to facilitate the development of wheat cultivars adapted to water-limitation by providing selection tools and genetic resources that would allow breeders to target favourable root traits. Seminal root angle (SRA) and seminal root number (SRN) are two traits that have been linked to root architecture, favouring water extraction from deep in the soil and late in the season in terminal moisture stressed environments.

In Chapter 3, the development and validation of a simple yet innovative phenotyping method is described. The method is based on transparent plastic pots used to evaluate SRA in five-day-old wheat seedlings and SRN in 11-day-old wheat seedlings under controlled conditions. Compared to previously reported methods, the technique developed here is low-cost and high-throughput. Using this method, significant genetic variation for both traits was identified, and the traits also exhibited high heritability which is important if they are to be manipulated in a breeding program. The method provides precise and reproducible phenotypic information at early growth stages, out-of-season, and in a more homogeneous environment than the field. These features make the method more suited to large-scale characterisation of seedling root traits in crop improvement programs than previously reported methods.

The potential to manipulate a root architectural trait in early generations of wheat breeding was demonstrated in Chapter 4. Genetic diversity for SRA was identified in a panel of wheat lines including lines adapted to Australian cropping environments. Alleles for narrow and wide SRA were rapidly introgressed into elite cultivars via rounds of phenotypic selection using the clear-pot system described in Chapter 3. This resulted in a clear shift in population distribution and allele frequencies in the resulting tail populations after only two cycles of selection. Further, selection using molecular markers successfully distinguished groups of

lines differing for SRA. This demonstrates that breeders could rapidly enrich their germplasm with favourable alleles for root system architecture (RSA) or eliminate undesirable gene combinations early in the breeding cycle by applying direct phenotypic selection or through marker-assisted selection (MAS).

An investigation of the genetic control of seminal root traits is reported in Chapter 5. Three nested association mapping (NAM) populations were developed and used to investigate alleles from multiple sources. This large multi-reference parent nested association mapping (MR-NAM) population captured high genetic recombination and high genetic diversity from a panel of founders. These founders were crossed with three elite spring wheat cultivars, each having adaptation to one of the three major wheat cropping regions of Australia. Genome wide association mapping studies (GWAS) identified a total of 30 QTL for SRA and SRN, among which eight were overlapping with genomic regions previously identified in wheat. Effects of QTL alleles were estimated across the three elite genetic backgrounds, revealing complex genetic controls for both traits. Identification of key QTL that were consistent across NAM populations and across studies will aid breeders to combine desirable traits in elite germplasm adapted to water-limited environments.

The implications for how wheat breeders could incorporate this new knowledge and these breeding selection tools into their breeding programs are discussed in this final chapter. Limitations of the study, as well as strategies to accelerate the development of superior genotypes, are further discussed.

Incorporating selection tools for root traits into breeding programs

Crossing designs

Selection of parental lines to be used in crosses is an important decision for plant breeders because it provides genetic variability in segregating progeny populations. Breeders design crosses to either recombine alleles already present in the genepool or to introduce new alleles from exotic germplasm. The tools developed in this study could be incorporated into breeding programs to select parental lines from diverse genetic resources, such as synthetic-hexaploid wheats, landraces, and modern breeding lines. Synthetic-hexaploid wheats and landraces represent an important source of untapped genetic diversity that can be used to broaden the genetic basis of existing elite germplasm for forward selection (Reynolds *et al.*, 2007; Feuillet *et al.*, 2008). Hence, wheat breeders could use the clear-pot

method developed in Chapter 3 to characterise and select fixed lines with desirable root characteristics. Alternatively, wheat breeders could use QTL-associated markers identified in Chapter 5 to select fixed lines with complementary genes for desired RSA. Breeders could then cross the selected fixed lines using single cross, three-way cross, or backcross schemes, to produce segregating populations.

Early generation selection

By performing selection in early generations, plant breeders can eliminate lines with undesirable gene combinations and enrich initial segregating populations with desired alleles. In Chapter 4, we demonstrated that population distribution and allele frequencies for SRA could be shifted in early generations. Further, high heritability observed for both SRA and SRN in Chapter 3 and Chapter 5 confirmed that the genetic component determining these traits was sufficient to enable phenotypic selection or MAS in early generations. Hence, breeders could perform phenotypic screening using the clear-pot method developed in Chapter 3 or apply MAS for the QTL-associated markers identified in Chapter 5 in segregating populations. Breeders could then advance a smaller 'superior' set of inbred lines to the more expensive field-testing phase. Further, with repeated cycles of selection, breeders could eventually fix some key genes associated with desired RSA in their breeding germplasm.

Marker-assisted backcrossing

Marker-assisted backcrossing (MABC) allows a small set of QTL from donor parents to be introgressed into elite germplasm, while minimising transfer of undesirable genes from the donors. MABC has been successfully used in rice (*Oryza sativa* L.), for example to introgress four QTL for deep rooting into elite cultivar IR64 (Shen *et al.*, 2001), and four QTL for root length and thickness into elite cultivar Kalinga III (Steele *et al.*, 2006). However, a potential limitation of using MABC is when multiple QTL control the expression of complex traits (Mir *et al.*, 2012). Hence, it is unlikely that MABC would be efficient for the 30 QTL for root traits identified in Chapter 5. However, a QTL prioritization analysis could be conducted to select highest priority target QTL, and used for MABC to develop lines with improved RSA. Prioritizing QTL consists of examining the value of different combinations of QTL to find those with the highest breeding value, in terms of size of the additive effects, and consistency of QTL effects across genetic backgrounds, and on grain yield across a broad range of environments (Collins *et al.*, 2008; Tuberosa, 2012; Maccaferri *et al.*, 2016).

Strategies to determine the priority of including any particular QTL, or combinations of QTL, in particular environments and genetic materials are further discussed in the section below.

Marker-assisted recurrent selection

Marker-assisted recurrent selection (MARS) is preferred for targeting multiple QTL in comparison to MABC because it gradually increases the frequency of favourable alleles by inter-mating selected individuals in each selection cycle (Eathington *et al.*, 2007; Bernardo, 2008). MARS could be used for pyramiding QTL, such as those identified in Chapter 5, to develop superior lines. MARS could also be used to test the additive effects of specific combinations of genes as part of the process of selecting the highest priority target QTL and QTL haplotypes as discussed above. In Chapter 5, we report on identification of multiple QTL for SRA and SRN, with both positive and negative effects. Thus, many combinations of alleles for the genes are possible. Further, some QTL for SRA overlapped with QTL for SRN. Therefore, it is possible that genes are interacting with each other with epistatic, additive, synergistic, and/or antagonistic effects. By stacking multiple QTL for RSA, breeders could identify favourable gene combinations for a desired phenotype, and use this information to select parental lines for crosses, screening of the offspring, or genomic selection as discussed below.

Genomic selection

With genomic selection (GS), a prediction model is used to predict phenotypes for traits that are controlled by multiple genes with small effects (Meuwissen *et al.*, 2001; Heffner *et al.*, 2009). As opposed to MAS, GS takes into account whole-genome profiling rather than selected QTL-associated markers. Therefore, known marker-trait associations are not required. However, recent studies have shown that higher prediction accuracies were achieved when markers linked to large or moderate effect QTL were included in the prediction model as fixed effects (Zhao *et al.*, 2013, 2014; Rutkoski *et al.*, 2014). Thus, wheat breeders could include information on QTL such as those identified in Chapter 5, or the subset of highest priority target QTL, in their selection approach. This strategy seems more appropriate for breeding purposes: with the implementation of routine whole-genome profiling in breeding programs, prediction models for any trait can be applied for GS (Poland and Rutkoski, 2016). Hence breeders could effectively combine GS for multiple desirable traits such as yield, disease resistance, agronomic performance, quality, and optimal RSA, at no additional direct cost.

Validation of seminal root traits in a breeding context

Limitation of the study

In this study, we showed SRA and SRN were highly heritable, presented a high level of genetic variation indispensable for breeders, and were suitable for rapid and cost-effective phenotypic and molecular selection. But will breeders adopt selection for such traits? Richards (1996) suggests that breeders will remain unconvinced until there is evidence suggesting they could make important yield gains by selecting for specific traits. Hence, one of the main limitations of this study is that the value of seminal root traits in diverse environments and management scenarios were not investigated. Further, for Wissuwa (2009), having successfully identified QTL associated with the trait of interest is not the major bottleneck to convince breeders of their usefulness in target environments, but rather the lack of relevant QTL in terms of breeding values. Confirmation of effects associated with QTL and molecular genetic approaches to identify underlying genes could not be conducted within the scope or the present study. The factors are discussed in the sections below.

Root traits and genetic correlation with yield

Many environmental factors can influence the shape and the size of the root system (Fang *et al.*, 2009). For example, root growth and distribution at depth can be influenced by soil temperature (Onderdonk and Ketcheson, 1973), soil structure (White and Kirkegaard, 2010), soil compaction (Jin et al., 2015; Ramalingam et al., 2017) and soil nutrient content (Bonser et al., 1996). As a consequence, the RSA in seedlings may not always be representative of the mature plant (Watt *et al.*, 2013). Thus, proof of concept in the field is required to determine the value of specific seminal root traits in diverse environments which vary in soil type and rainfall patterns.

Previous studies in cereal crops showed that narrow root angle and high number of roots in seedlings are associated with deeper and more compact RSA at the adult stage (Nakamoto *et al.*, 1991; Oyanagi *et al.*, 1993, 2001; Manschadi *et al.*, 2008). Other studies across cereal crops showed that mature RSA is associated with water extraction at different depths and yield in water-limited environment, particularly under terminal moisture stress (Ludlow and Muchow, 1990; Tuberosa *et al.*, 2002*b*,*a*; de Dorlodot *et al.*, 2007). However, few studies have identified a direct link between root traits in seedlings and final yield. The most striking example is found in rice, where a major QTL for root growth angle, *DEEP ROOTING 1*

(*DRO1*), was directly associated with rooting depth and improved yield under water-limited environments.

In this thesis, association between root traits measured at the seedling stage in controlled environment conditions, mature RSA, and yield, were not investigated. However, some genetic resources developed in this project offer great opportunity to further investigate this link. Tail populations developed in Chapter 4 are populations where divergent selection has been practised for SRA. Mature RSA of tail populations selected for narrow and wide SRA could be compared using soil-filled root observation chambers (Manschadi *et al.*, 2008), high-throughput 'shovelomics' (Trachsel *et al.*, 2011), or soil coring (Wasson *et al.*, 2014). Further, tail populations could be assessed in multi-environment trials for yield and surrogate traits for deep rooting, such as stay-green and/or canopy temperature depression using high-throughput multi-sensor systems in the field (Bai *et al.*, 2016). Such field experiments could also be conducted with the MR-NAM population described in Chapter 5. The MR-NAM resource presents high phenotypic variation for both SRA and SRN. The population also has a relevant agronomic window due to moderate selection pressure applied during development. This will facilitate realistic comparison between the yield and phenotypic expression of other adaptive traits, by reducing confounding effects from variation in height and flowering date, and by limiting competition between neighbouring plots in the field trials. GWAS can also be performed in the MR-NAM population to dissect yield and drought-adaptive traits, and compare overlapping genomic regions influencing yield and seminal root traits.

Benefit of soil exploration in a farming system

The capture of subsoil water by deeper wheat roots can make a valuable contribution to yield (Kirkegaard *et al.*, 2007; Christopher *et al.*, 2008; Lilley and Kirkegaard, 2008). Simulation studies have investigated the benefits of deeper wheat roots in a range of environments where water was available in the deep soil layers (King *et al.*, 2003; Asseng and Turner, 2007; Semenov *et al.*, 2009; Manschadi *et al.*, 2010; Lilley and Kirkegaard, 2011). These investigations indicate that overall, wheat varieties with faster and more efficient roots provide yield benefits in most European and Australian cropping environments, and are rarely predicted to result in yield reduction. At sites with shallower soils as encountered in western and southern Australia, the benefits of more extensive root systems could be negligible but rarely are they adverse.

Most previous simulation studies assumed crops were sown into fully wet profiles. However, a range of environmental factors such as rainfall distribution and soil type can influence the soil water available at sowing. For example poor soil moisture retention or insufficient rainfall to replenish soil moisture at depth can leave the soil in a drier state. In this case, benefits of deep rooting might be reduced. Management factors, such as rotation sequence, weed control, and timely sowing, can also influence the soil water reservoir, overriding or enhancing the predicted yield benefits arising from deeper rooting (Kirkegaard *et al.*, 2007; Lilley and Kirkegaard, 2011). For example, crops with deeper roots extract more water, but may also remove soil moisture that would otherwise be available for subsequent crops, reducing the predicted long-term system benefit (Lilley and Kirkegaard, 2016). Hence, investments into deep rooting selection need to be targeting towards environments and management scenarios for which the largest yield benefits will arise. Again, the selected tails and MR-NAM populations developed in this study both offer the opportunity to identify those environments and optimal management practices.

Root function and impact on crop performance

Roots play a key role in water uptake but also provide essential functions for nutrient uptake, anchoring the plants to the soil, and interacting with organisms in the rhizosphere. Depending on target environmental stresses, different root architectures might be desired. For example, selection for narrower growth angle may have some benefit to increase access to deep soil water (Wasson *et al.*, 2012), but selection for wider growth angle may increase access to phosphorus in the surface soil (Lynch and Brown, 2001; Lynch, 2013). Hence, better knowledge of root functional traits and how traits are related to the whole plant strategies would provide the opportunity for breeding programs to design optimized root ideotypes for crop improvement in a range of environments (de Dorlodot *et al.*, 2007; Hammer *et al.*, 2009; Lynch, 2013; White *et al.*, 2013; Brown *et al.*, 2013; Meister *et al.*, 2014).

Studies have been carried out to characterize genes and describe the genetic control of RSA in maize (*Zea mays* L.), rice, soybean (*Glycine max*), and wheat (Meister *et al.*, 2014). For example, fine mapping and cloning of *DRO1* showed that the gene functions downstream of the auxin signalling pathway, leading to gravitropic bending of rice roots (Uga *et al.*, 2013). Positional cloning can help to understand basic biological mechanisms by isolating and testing candidate genes segregating with the QTL of interest (Salvi and Tuberosa, 2005). The strategy of using heterogeneous inbred families (HIFs) has been

widely used in QTL fine mapping and cloning (Tuinstra *et al.*, 1997). In Chapter 5, we developed 612 $F_{4:5}$ NAM lines, with an expected heterozygosity of 6%. This level of heterozygosity offers the opportunity to identify HIF, and to rapidly establish near isogenic lines (NILs) that differ at a specific QTL. The use of NILs minimizes the influence of the genetic background. These could be a valuable resource to validate the roles of candidate genes in diverse environments. Further, in most studies, genes controlling root traits also had an effect on shoot traits, confounding the evidence for benefits of root variation (Ma *et al.*, 2012; Bian *et al.*, 2012). Hence, it is necessary to determine the combinations of shoot and root traits that benefit whole plant productivity to identify optimal root phenotypes for crops to be grown in specific environments.

Breeding wheat for the future

Breeding wheat for drought adaptation

While seminal root traits may influence access to water in later stages of the crop cycle, there are many other physiological traits that influence water use, and consequently yield under water-limited conditions (Monasterio *et al.*, 2001; Monneveux *et al.*, 2012; Lopes *et al.*, 2014). For example, other traits related to the RSA may affect water use, such as root to shoot ratio, root hair development, root branching and inter-branch root length (Wasson *et al.*, 2012; Comas *et al.*, 2013). Other physiological traits contributing to drought-adaptation include flowering and maturity dates, plant height, traits relating to pre-anthesis growth such as early vigour or tillering, integrated traits such as canopy temperature, traits relating to water-use efficiency such as transpiration efficiency or its proxy carbon isotope discrimination (Blum *et al.*, 1989; Villegas *et al.*, 2000; Condon *et al.*, 2004; Olivares-Villegas *et al.*, 2007; Rebetzke *et al.*, 2012*b*, 2013), and stay-green (Christopher *et al.*, 2008, 2014, 2016*b*). Hence, breeding for several of these traits at the same time should be considered, especially given the fact that there can be some 'trade-off' between these traits. For example, as roots and shoots are able to communicate through complex signalling pathways, selection for optimal RSA may also impact growth and development of the above-ground parts of the plants. Multi-trait selection can be achieved through the development of high throughput phenotyping methods as well as the use of selection indices.

Increasing genetic gain for yield

Crops are constantly exposed to biotic and abiotic factors that reduce yield and quality, threatening food security worldwide. Plant breeders must constantly respond to these changes. For instance, the rapid evolution of pathogens forces plant breeders to continually search for new sources of resistance genes. Anticipated effects of higher temperatures and increased salinity due to climate change also provide additional challenges for plant breeders and geneticists to ensure yield stability in diverse environments. Furthermore, industry and consumer preferences also change, leading to variation in quality requirements, as we have seen with the preference for gluten free diets, mostly driven by fashion. Thus, combining multiple desired traits to rapidly develop improved cultivars is necessary to meet the growing demand.

Here, we developed a MR-NAM population, a powerful pre-breeding platform that combines identification of new sources of genetic improvements with rapid introgression into commercial cultivars. The population development pipeline is rapid and efficient, hence the MR-NAM population could be rapidly expanded. Additional crosses could be developed in parallel, using breeding lines or cultivars as new founders or as new reference parents, to increase the size, genetic diversity, and therefore the power of the population. To harness more genetic diversity or novel alleles, unadapted germplasm such as synthetic-hexaploid wheats or landraces could be used as new founders, in combination with a backcrossing strategy to reduce the frequency of non-adaptive alleles. This approach was proposed by Jordan et al. (2011) to increase the genetic diversity of elite Australian sorghum (*Sorghum bicolor* L.) germplasm, while retaining adaptive traits including height and maturity. Combined with high-throughput phenomics platforms, we anticipate this powerful genetic resource will lead to important discoveries in linking genotype to phenotype, and will assist wheat breeders to unlock the genetic potential of wheat in Australia and world-wide.

Conclusion

Breeding for root traits has been hampered by the lack of efficient high throughput phenotyping methods and relatively poor understanding of the genetic controls. This thesis provides new tools to empower wheat breeders to target specific root traits, including a high-throughput and cost-effective phenotyping method, identification of useful sources for extreme seminal root traits, knowledge of the genetic controls, and molecular markers

associated with QTL in multiple genetic backgrounds. The research also provides valuable genetic resources, such as populations where divergent selection has been practised for seminal root angle and a powerful pre-breeding platform, which will help breeders determine the preferred root ideotypes for various target environments.

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Appendices

Appendix 1: Average BLUPs per cultivar of the two clear pot experiments Clear_1 and Clear_2 and the two growth pouch experiments Pouch_1 and Pouch_2.

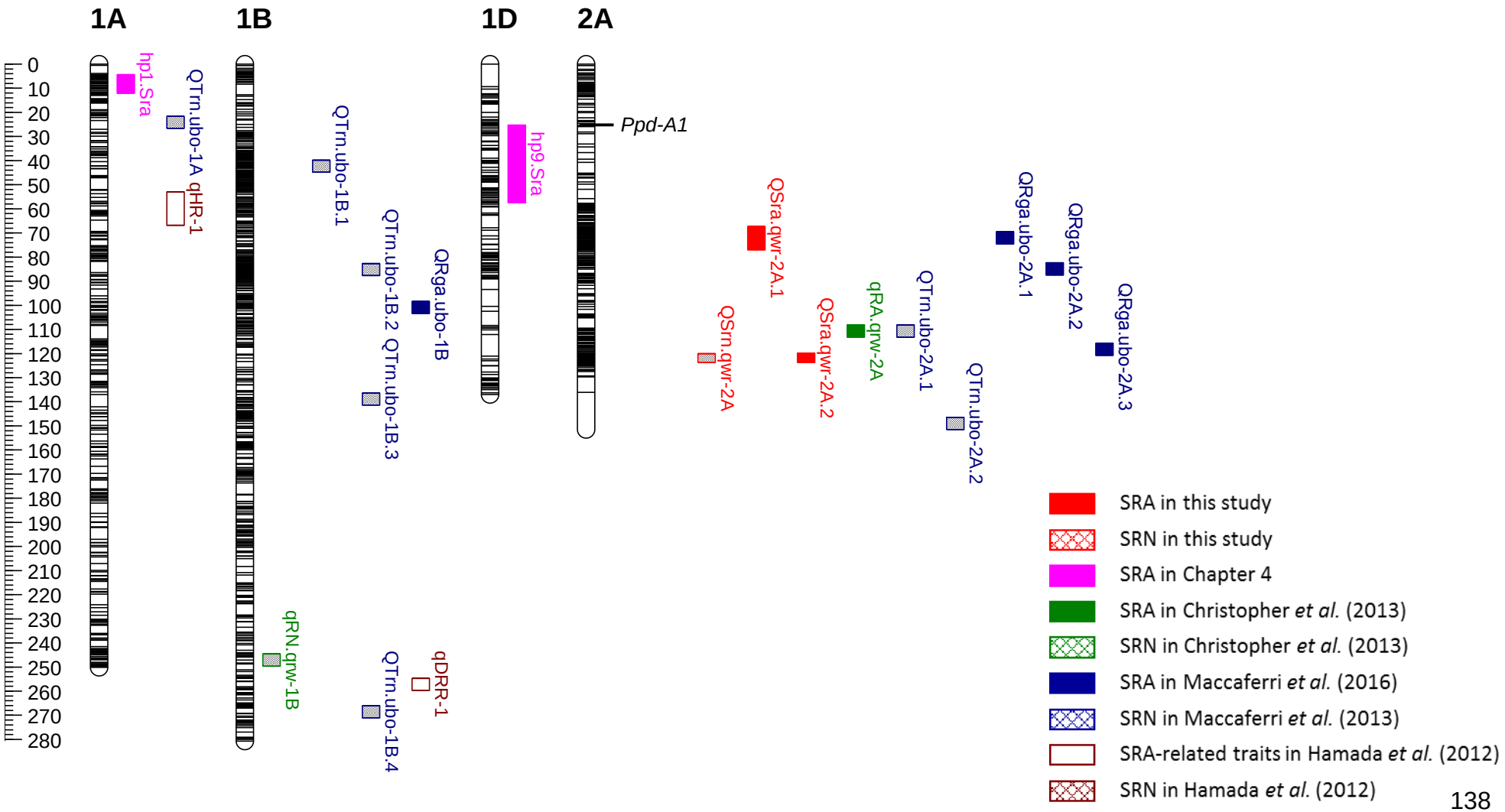
	Seminal root angle				*Seminal root number*					
Cultivar	**clear_1**	**clear_2**	**pouch_1**	**pouch_2**	**clear_1 imaged**	**clear_2 imaged**	**clear_1 extracted**	**clear_2 extracted**	**pouch_1**	**pouch_2**
Babax	77	74	105	110	3.6	3.4	3.9	3.5	3.9	3.9
Baxter	73	73	104	111	3.8	3.5	4.6	4.4	3.7	4.1
Chara	62	69	91	126	3.5	4.0	3.8	4.7	4.0	4.0
Dharwar Dry	73	76	107	116	3.6	3.5	4.7	4.1	3.9	4.2
Diamondbird	84	84	106	124	3.3	3.9	3.8	4.2	3.9	3.9
EGA Gregory	85	77	107	117	3.8	3.3	4.2	4.1	3.7	3.9
EGA Hume	77	75	105	124	3.7	3.5	4.6	4.0	3.8	4.1
EGA Wedgetail	51	69	102	106	3.5	3.5	3.5	3.7	3.6	3.7
EGA Wentworth	71	70	106	96	3.6	3.2	4.1	3.5	3.6	4.0
Frame	75	78	97	118	3.7	3.7	4.6	4.7	4.1	4.1
Giles	59	67	97	107	3.7	3.8	4.6	4.7	3.9	4.2
Hartog	86	82	108	126	3.3	3.2	3.7	3.6	3.7	3.7
Janz	62	75	106	122	3.4	3.7	3.4	3.6	3.6	3.8
Krichauff	81	74	102	122	3.6	3.4	4.4	3.5	3.7	3.7
Lang	60	67	95	116	3.8	3.9	4.7	5.0	3.9	4.1
Leichhardt	79	83	101	117	3.8	3.8	4.8	4.8	3.8	3.9
Petrie	82	84	98	111	3.6	3.7	4.6	4.7	3.8	4.1
SeriM82	87	81	110	125	3.5	3.6	4.2	4.4	3.8	4.0
Silverstar	81	79	109	116	3.7	3.6	4.5	4.3	3.6	3.9
Sunco	78	77	103	108	4.0	3.7	4.9	4.3	3.8	4.1
Sunvale	69	71	104	107	3.9	4.1	4.8	4.5	4.3	4.1
Ventura	83	79	108	126	3.5	3.3	4.0	3.8	3.6	4.0
Wyalkatchem	84	82	98	117	3.6	3.4	4.2	3.9	3.6	3.9
Yitpi	83	77	105	122	3.8	3.7	4.6	4.4	3.8	4.0

Appendix 2: Number of polymorphic markers after filtering to remove markers with less than 10% occurrence within each family (bold) and in common between families

NAM families	*Mace/ Dharwar Dry*	*Mace/ Drysdale*	*Mace/ Westonia*	*Mace/ ZWW10-50*	*Scout/ Dharwar Dry*	*Scout/ Drysdale*	*Scout/ RIL114*	*Scout/ SeriM82*	*Scout/ SB062*	*Suntop/ Dharwar Dry*	*Suntop/ EGA Gregory*	*Suntop /SB062*	*Suntop/ EGA WYLIE*	*Suntop/ ZWB10-37*	*Suntop/ ZWW10-128*
Mace/Dharwar Dry	**8,346**	4,345	3,734	4,312	3,933	3,087	3,103	3,259	2,505	4,999	3,244	3,491	4,013	3,792	2,917
Mace/Drysdale		**7,549**	3,548	4,588	2,647	3,576	2,879	2,883	2,361	3,476	3,001	3,302	3,601	3,630	2,889
Mace/Westonia			**6,300**	3,570	2,485	2,248	2,545	2,389	1,831	3,165	2,699	2,775	2,989	2,735	2,302
Mace/ZWW10-50				**7,780**	2,806	2,995	3,117	3,007	2,571	3,740	3,232	3,598	3,564	3,772	3,001
Scout/Dharwar Dry					**6,986**	3,313	3,357	3,372	2,810	4,122	2,471	2,811	2,897	3,112	2,376
Scout/Drysdale						**6,989**	3,461	3,395	3,135	3,296	2,710	3,068	3,008	3,145	2,465
Scout/RIL114							**7,028**	3,422	2,624	3,218	2,786	2,900	2,980	2,973	2,424
Scout/SeriM82								**7,732**	2,664	3,634	3,327	3,543	4,312	3,484	2,956
Scout/SB062									**6,229**	2,595	2,362	3,599	2,507	3,214	2,192
Suntop/Dharwar Dry										**8,998**	4,549	4,869	4,777	5,106	4,053
Suntop/EGA Gregory											**7,440**	4,048	4,702	4,422	3,539
Suntop/SB062												**8,527**	4,187	5,940	4,737
Suntop/EGA WYLIE													**8,263**	4,399	3,628
Suntop/ZWB10-37														**8,733**	4,370
Suntop/ZWW10-128															**6,677**

Appendix 3: Quantitative trait loci (QTL) for seminal root traits identified in wheat and projected onto the diversity array technology (DArT) consensus map

A total of 77 QTL were sourced for seminal root angle (SRA) and seminal root number (SRN) from three discovery papers (Hamada *et al.*, 2012; Christopher *et al.*, 2013; Maccaferri *et al.*, 2016), along with the 30 QTL identified in Chapter 5, and the 13 hotspots identified in Chapter 4. Confidence intervals adjusted to 5 cM for display purpose for QTL spanning less than 5 cM. Key agronomic genes and filtered markers are represented by a single line. Marker distance in centimorgan is indicated by the scale on the left.



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Ppd-B1
QSra.qwr-2B.1
QSrn.qwr-2B.1
QSrn.qwr-2B.2
QSra.qwr-2B.2
QSra.qwr-2B.3
hp2.Sra
hp10.Sra
qRA.qrw-2B
QTrn.ubo-2B.1
QTrn.ubo-2B.2
QRga.ubo-2B.1
QRga.ubo-2B.2
QRga.ubo-2B.3
QRga.ubo-2B.4
QRga.ubo-2B.5
qHR-2
QSrn.qwr-2D
hp3.Sra
qRN.qrw-3A
QTrn.ubo-3A.1
QTrn.ubo-3A.2
QRga.ubo-3A
SRA in this study
SRN in this study
SRA in Chapter 4
SRA in Christopher et al. (2013)
SRN in Christopher et al. (2013)
SRA in Maccaferri et al. (2016)
SRN in Maccaferri et al. (2013)
SRA-related traits in Hamada et al. (2012)
SRN in Hamada et al. (2012)

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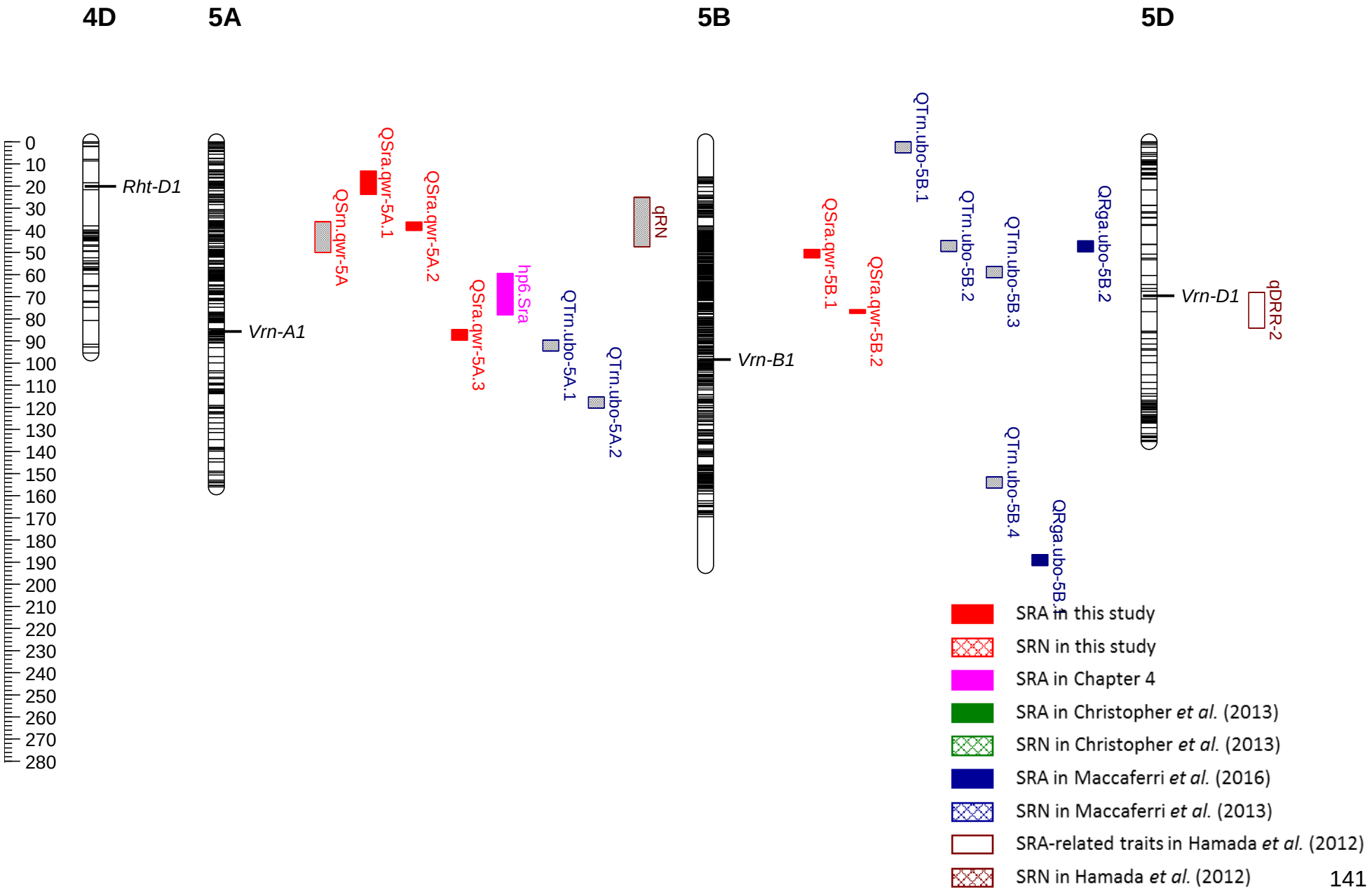
QSra.qwr-3B.1
QSra.qwr-3B.2
hp4.Sra
QTrn.ubo-3B.1
QTrn.ubo-3B.2
qRN.qrw-3B
QRga.ubo-3B
QSrn.qwr-3D
hp5.Sra
QSrn.qwr-4A
hp11.Sra
qRN.qrw-4A
QTrn.ubo-4A.1
QTrn.ubo-4A.2
QTrn.ubo-4A.3
QRga.ubo-4A
Rht-B1
QTrn.ubo-4B.1
QTrn.ubo-4B.2
QTrn.ubo-4B.3
QTrn.ubo-4B.4
QRga.ubo-4B.1
QRga.ubo-4B.2
QRga.ubo-4B.3
QRga.ubo-4B.4
SRA in this study
SRN in this study
SRA in Chapter 4
SRA in Christopher et al. (2013)
SRN in Christopher et al. (2013)
SRA in Maccaferri et al. (2016)
SRN in Maccaferri et al. (2013)
SRA-related traits in Hamada et al. (2012)
SRN in Hamada et al. (2012)


4D
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Rht-D1
Vrn-A1
QSrn.qwr-5A
QSra.qwr-5A.1
QSra.qwr-5A.2
QSra.qwr-5A.3
hp6.Sra
QTrn.ubo-5A.1
QTrn.ubo-5A.2
qRN
Vrn-B1
QSra.qwr-5B.1
QSra.qwr-5B.2
QTrn.ubo-5B.1
QTrn.ubo-5B.2
QTrn.ubo-5B.3
QTrn.ubo-5B.4
QRga.ubo-5B.2
QRga.ubo-5B.1
Vrn-D1
qDRR-2
SRA in this study
SRN in this study
SRA in Chapter 4
SRA in Christopher et al. (2013)
SRN in Christopher et al. (2013)
SRA in Maccaferri et al. (2016)
SRN in Maccaferri et al. (2013)
SRA-related traits in Hamada et al. (2012)
SRN in Hamada et al. (2012)

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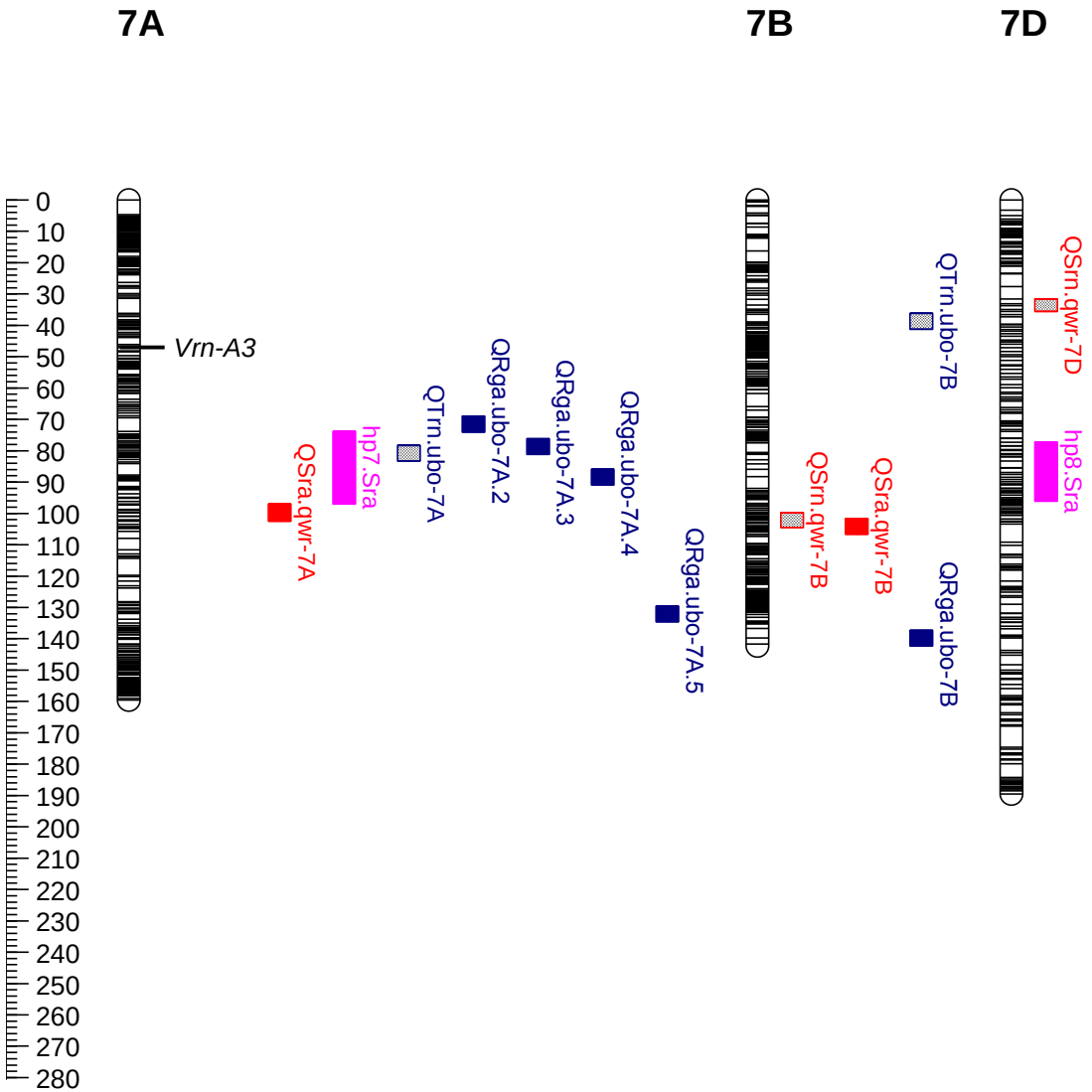
QSrn.qwr-6A
QSra.qwr-6A.1
QSra.qwr-6A.2
hp12.Sra
QRN.qrw-6A
QRA.qrw-6A.a
QTrn.ubo-6A.1
QRga.ubo-6A.1
QRga.ubo-6A.3
QRga.ubo-6A.2
QSra.qwr-6B.1
QSra.qwr-6B.2
hp13.Sra
QRA.qrw-6B.2
qRA.qrw-6B.1
QTrn.ubo-6B.1
QTrn.ubo-6B.2
QTrn.ubo-6B.3
QTrn.ubo-6B.4
QRga.ubo-6B.1
QRga.ubo-6B.2
QSrn.qwr-6D
SRA in this study
SRN in this study
SRA in Chapter 4
SRA in Christopher et al. (2013)
SRN in Christopher et al. (2013)
SRA in Maccaferri et al. (2016)
SRN in Maccaferri et al. (2013)
SRA-related traits in Hamada et al. (2012)
SRN in Hamada et al. (2012)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Vrn-A3
QSra.qwr-7A
hp7.Sra
QTrn.ubo-7A
QRga.ubo-7A.2
QRga.ubo-7A.3
QRga.ubo-7A.4
QRga.ubo-7A.5
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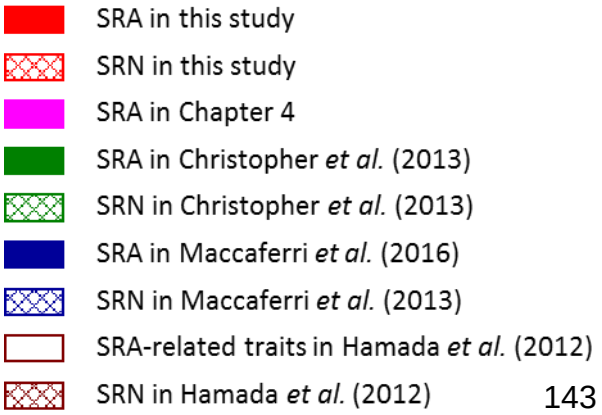
QTrn.ubo-7B
QRga.ubo-7B
QSrn.qwr-7D
hp8.Sra


SRA in this study
SRN in this study
SRA in Chapter 4
SRA in Christopher *et al.* (2013)
SRN in Christopher *et al.* (2013)
SRA in Maccaferri *et al.* (2016)
SRN in Maccaferri *et al.* (2013)
SRA-related traits in Hamada *et al.* (2012)
SRN in Hamada *et al.* (2012)